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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This report describes the effects of treatment of male Sprague-Dawley rats with low levels of the cholinesterase (ChE) inhibitors Sarin (0.5 LD50 s.c. 3 times weekly) and pyridostigmine bromide (PB, 80 mg/L in drinking water) alone or in combination for 3 weeks as compared with untreated controls. The work during this reporting period included analysis of locomotor activity (LA) and autonomic control of heart rate (HR), as well as the metabolism of acetylcholine (ACh) and choline (Ch), at 2, 4 and 16 weeks after exposure to the ChE inhibitors. HR, derived from electrocardiogram, and LA were studied by radiotelemetry of animals in their home cages 24 hrs a day, during one week. ChE inhibitors decreased HR and enhanced HR variability 2 weeks post-treatment, but the effects did not persist until later times. No treatment-related changes in LA were found.

The expected regional variations in ACh, ACh turnover, and D4Ch, similar to the distribution of other cholinergic markers, or related to characteristics of the blood-brain barrier were found, but ACh and Ch contents were altered only at two weeks post-treatment. No treatment-related effects were detected in ACh turnover.

In conclusion, changes in the variables under study were mainly observed 2 weeks after treatment but failed to persist until later intervals. Thus, these experiments do not support the hypothesis that exposure to low levels of cholinesterase inhibitors could induce persistent or delayed effects in control of heart rate and locomotion or cholinergic metabolism.

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INTRODUCTION.

Organophosphorus (OP) cholinesterase (ChE) inhibitors are among chemical weapons to which army personnel and civilians could be exposed, at symptomatic or sub-symptomatic doses. The carbamate ChE inhibitor pyridostigmine bromide (PB) has been fielded as a prophylactic treatment against OP ChE inhibitors by the US Armed Forces and used in the Persian Gulf War (Dirnhuber et al.,1979); (Leadbeater et al.,1985); (Koplovitz et al.,1992); (Kluwe et al.,1987); (Keeler et al.,1991). Although acute intoxication with OP ChE inhibitors and the protective effect of PB on this phenomenon have been extensively studied in animals (Ecobichon and Joy,1982); (Sidell,1974); (Chambers,1992), the potential long term harmful effects of low level (subsymptomatic) exposure to OP ChE inhibitors, alone or in combination with PB have received little attention. This is the objective that the present program intends to address.

In our experimental approach to this objective, we are evaluating the possible occurrence of delayed neurologic dysfunction after exposure of animals to PB or to doses of the OP cholinesterase inhibitor sarin, low enough to be free of acute toxic effects, alone or in combination with PB treatment.

During the past year, we have carried out experiments to complete the original program during a no cost extension from February 2004 to February 2005. The work included studies in two general areas, analysis of spontaneous activity and autonomic control of heart rate, and a comprehensive study of the metabolism of acetylcholine (ACh) and its precursor and degradation product, choline (Ch).

MATERIALS AND METHODS.

Animals.

Male Crl:CDBR Vaf/Plus Sprague-Dawley rats, weighing 250-300g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21 ± 2 °C) and humidity (50 ± 10%) controlled animal quarters maintained on a 12- h light-dark full spectrum lighting cycle. Laboratory chow and water were freely available. Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Materials.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs Inc. (Berkeley, CA). Sarin, obtained from the U. S. Army Edgewood Chemical and Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Animal dosing procedures were performed at the APG laboratory. Saline or sarin injection volume was 0.5 ml/kg subcutaneously. PB was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared twice weekly in tap water at a concentration of 80 mg/L and provided as drinking water to experimental groups for a three-week period.

Experimental groups: Separate sets of animals were studied 2, 4, and 16 weeks after treatment. Within every set, animals were divided into 4 treatment groups. Number of animals were, for the heart rate and locomotor activity experiments: 2 weeks post-treatment, control=6, PB=7, sarin=6, sarin+PB=7; 4 weeks post-treatment, control=8, PB=8, sarin=8, sarin+PB=6; 16 weeks post-treatment, control=8, PB=8, sarin=8, sarin+PB=6. In the case of ACh and Ch metabolism experiments: 2 weeks post-treatment, control=11, PB=11, sarin=13, sarin+PB=11; 4 weeks post-treatment, control=9, PB=11, sarin=10, sarin+PB=9; 16 weeks post-treatment, control=10, PB=11, sarin=10, sarin+PB=8.

The control animals received regular tap water as drinking water and were injected with saline. The PB group animals received PB in drinking water (80 mg/L) and were injected with saline. The sarin group animals received tap water and were injected with sarin (62.5 ug/kg, sc, equivalent to 0.5 LD50). The sarin+PB group of animals received PB in drinking water and were injected with sarin at the regimen described above. PB in drinking water was provided continuously to the PB and PB+sarin groups starting on Monday morning at 08:00 hours. At 09:00 hours that Monday morning, injection of either saline (0.5 ml/kg, sc) or sarin (62.5 ug/kg, sc) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week for three weeks in groups of four animals per dose. PB in drinking water was terminated and switched to regular tap water at 17:00 hours on Friday of the third week. Animal dosing procedures were performed at the APG laboratory. All animals were then shipped to the VA GLA laboratory location, where the planned studies in these animals were performed 2, 4 and 16 weeks after sarin, sarin + PB, PB, or control treatments. A period of one week was allowed after animals were transferred before starting experimentation.

Implantation of telemetry transducers and recording of electrocardiogram and locomotor activity: Radiotelemetry transmitters, specifically designed for rats (Data Sciences International TL10M3 F50 EEE) were implanted subcutaneously on the back of the animal, approximately just below the shoulder blades, using aseptic technique. The transmitter weighs 11.5 g., and it has a volume of 5.5 cm^3. A 2 cm skin incision was performed between the scapulae. Animals were anesthetized by exposure to 2.5% halothane in air in a closed plexi-glass chamber with continuous flow of gas from an anesthesia machine. After 2-3 minutes the animal was transferred to a table provided with a heating pad, and a maintenance concentration of halothane (1.5%) was given by mask throughout the surgical procedure. A scavenging system (Fluosorb) prevented excess halothane from reaching the environment. The concentration was raised if withdrawal to painful stimulation was observed.

The radiotelemetry implant was placed on a pocket fashioned by blunt dissection of the subcutaneous space at the site. One pair of leads was set up for recording of electrocardiogram (ECG) by suturing one of them with 5-0 polyvinyl material to the subcutaneous tissue over the right scapula and the other one at the level of the heart apex. The skin incision over the radiotelemetry implant was closed with 5-0 polyvinyl suture material.

Anesthesia was discontinued after surgical wounds were sutured. The condition of the animal was monitored frequently during the post-operatory period.

In order to analyze circadian variations of heart rate (HR) and locomotor activity (LA), as well as heart rate variability, ECG and LA were recorded every 30 minutes, for an interval of 300 seconds, during seven consecutive days, starting four days after implantation of telemetry units. Using the Data Sciences software, the time of occurrence of each heartbeat was extracted from the raw ECG and a 300 seconds time series of consecutive inter-beat intervals (RR

intervals) was constructed to allow subsequent time domain and frequency domain measurements.

Power Spectrum analysis of HR fluctuations: For this analysis, the time series of RR intervals was re-sampled at a rate of 10 Hz and subsequently the frequency spectrum was calculated using a Fast Fourier Transform. The spectrum was further averaged over consecutives non-overlapping windows of 128 data points (leading to a window length of 21.33 seconds and a lowest frequency resolved of 0.05 Hz).

Data are presented as cumulative power over the following frequency bands: total (between 0.05 and 5 Hz), low frequency (between 0.26 and 0.75 Hz), high frequency (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (Pereira de Souza et al.,2001).

Concentrations of ACh, Ch, their deuterated variants, and ACh turnover in brain tissue.

Animals were anesthetized with halothane/N_2O, and a polyethylene (PE50) catheter was inserted in a femoral vein through a cutdown on the inguinal area. After suturing the skin, anesthesia was discontinued and the animals were positioned in a restraining device adapted for introduction into the animal chamber of the microwave fixation apparatus (Gerling Biostat, nominal power = 5kW). Fifteen minutes after discontinuation of anesthesia, Deuterium labeled Ch ($^2H_4$)-Ch (20 μmol kg^{-1} of the tosylate salt in saline) was injected. Following one minute, a bolus of 50mg/kg thiopental was infused intravenously and the microwave power turned on for 1 second/ 100g. body mass. The brain was rapidly removed, and cooled. Six regions were dissected: hippocampus, infundibulum, mesencephalon, neocortex, piriform cortex, and striatum. These tissue fragments were homogenized in ice cold 15% 1N formic acid, 85% acetone for analysis of ($^2H_0$)- and ($^2H_4$)-Ch (D0Ch and D4Ch respectively) and ($^2H_0$)- and ($^2H_4$)-ACh

(D0ACh and D4ACh respectively) by GCMS, using ($^2H_9$)-Ch and ($^2H_9$)-ACh contained in the tubes in precisely known amounts as internal standards. The homogenate was centrifuged and the supernatant transferred to clean centrifuge tubes and extracted with diethyl ether. The aqueous residue remaining after the ether extractions was used for GCMS determination of Ch and ACh, which is currently capable of detecting 10^{-13} mole of Ch and ACh (Jenden et al.,1973). First, these compounds are extracted in the following way: to the aqueous residue mentioned above is added an equal volume of 1M TAPS buffer, pH 9.2 and 2 volumes of 1mM dipicrylamine in methylene dichloride. After shaking and centrifuging, the aqueous phase is discarded; the organic phase is transferred to a clean centrifuge tube and evaporated to dryness. Then a solution of silver p-toluenesulfonate (5mM in acetonitrile:0.5 ml) and 50 µl propionyl chloride are added, shaken, allowed to stand at room temperature for 5 minutes and evaporated to dryness. N-demethylation is carried out with sodium benzenethiolate in anhydrous butanone; this is followed by two liquid partition steps to separate tertiary amines from neutral and acidic compounds, and an aliquot of the residue is injected into the GCMS system. Ions at m/e 58, 64 and other masses which may be of interest are monitored.

ACh turnover (AChT) was calculated as the ratio of D4ACh to the Ch molar ratio (D4Ch/(D0Ch+D4Ch)).

Statistical analysis: HR calculated from RR intervals was averaged every hour. A database consisting of seven days of HR hourly averages for every animal was analyzed performing repeated measures ANOVA with within factor "day" (seven levels) and between factor "treatment" (four levels) for every hour of the day. HR power spectrum was calculated for the third day of recording, every 2 hrs. Statistical analysis consisted of ANOVA with factor

"treatment" (four levels) for every 2 hr interval. Significance level for F ratios and multiple comparisons among treatment groups was set at 0.05 to establish significance.

Variables in ACh and Ch metabolism were studied by ANOVA with factors treatment, region of brain, and time post-treatment. Individual ANOVAs for every time post-treatment were also performed.

RESULTS

Heart rate and locomotor activity: The analysis of HR and locomotor activity dynamics conducted during a period of one week, with telemetry measurements of these variables averaged every hour through 24 hrs every day, indicated wide fluctuations of both variables throughout the day. Lights were turned on at 7:00 and off at 19:00 hours. ANOVA of HR values indicated significant effects for the factors treatment, and hour of day at all intervals after treatment. Maximal levels of HR were observed during the night and minimal during daylight hours (Figs 1-3, top panels).

Since HR correlates under most circumstances with the magnitude of physical activity, in this case estimated by the radiotelemetry signal level that codes for rate of displacement of animals inside their home cages (LA), it is important to examine the fluctuations in this variable to aid in the interpretation of circadian HR changes and the effects of treatments. Changes in LA recorded by telemetry simultaneously with HR are shown in Figs 1-3 (bottom panels).

It is obvious that in general the levels of HR tend to parallel the levels of LA in synchrony with the light/darkness cycle. Since rats are nocturnal animals, LA and HR were maximal during the dark period. The changes induced by treatments on the magnitude of these variables were not similar however. In the case of the groups studied 2 weeks after treatment, the differences in HR among treatment groups were not paralleled by LA. In fact, no significance between the treatment means of LA was found at any hour of the day (Fig 1, bottom panel). The same was true for the 4 and 16 week post-treatment times (Figs 2 and 3, bottom panels).

HR Power spectrum analysis (Figs 4-6) indicated that two weeks post-treatment, the sarin+PB group

showed increases in total and high power at 20 and 22 hrs, and low frequency power at 18, 20 and 22 hrs, while the ratio of low to high frequency power decreased at 20 and 22 hrs. The sarin group showed a decrease of low to high frequency power ratio at 20 hrs (Fig. 4).

Lesser changes were observed four weeks post-treatment, when low frequency power and the low to high power ratio decreased at 10 and 12 hrs, and 12 hrs respectively in the PB group (Fig.5).

At 16 weeks post-treatment, low to high frequency power decreased at 14 hrs in the PB group and high frequency power increased in the sarin+PB group at 12 hrs (Fig.6).

Tissue concentrations of ACh, Ch, their deuterated variants and the levels of ACh Turnover.

Factorial ANOVA (Tables 1-5) indicated significant effects ($p<0.05$) of region for all cholinergic variables studied. However, the patterns of regional variations were different for every variable that were ranked (from higher to lower mean values across all treatments and times post-treatments) as follows:

D0ACh: Striatum>Piriform>Mesencephalon >Hippocampus>Infundibulum=Neocortex.
D4ACh: Striatum>Hippocampus=Piriform=Neocortex>Mesencephalon>Infundibulum.
D0Ch: Infundibulum>Piriform=Striatum>Mesencephalon=Hippocampus=Neocortex.
D4Ch: Infundibulum>Piriform>Neocortex>Mesencephalon>Hippocampus=Striatum
ACh Turnover: Striatum>Hippocampus>Neocortex=Piriform=Mesencephalon.

These patterns were similar under all treatments, demonstrated by the lack of significant interaction of region and treatment in individual ANOVAs at every time post-treatment (Tables 6-8).

Levels of ACh turnover in Infundibulum were too low to be accurately resolved.

Table 1: Analysis of Variance for D0ACh.

Analysis of Variance Table

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Treatment	3	238.5345	79.5115	0.80	0.494423
B: Weeks	2	654.1577	327.0789	3.29	0.037927*
AB	6	1540.853	256.8088	2.58	0.017649*
C: Region	5	213400.6	42680.12	429.13	0.000000*
AC	15	1376.557	91.77043	0.92	0.538332
BC	10	720.723	72.0723	0.72	0.701592
ABC	30	624.2442	20.80814	0.21	0.999999
S	651	64747.36	99.45831		
Total (Adjusted)	722	289317.4			
Total	723				

* Term significant at alpha = 0.05

Table 2: Analysis of Variance for D4ACh.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Treatment	3	1.211608	0.4038692	1.24	0.293908
B: Weeks	2	0.9733984	0.4866992	1.50	0.224908
AB	6	5.554863	0.9258105	2.84	0.009657*
C: Region	5	92.99435	18.59887	57.15	0.000000*
AC	15	2.444297	0.1629532	0.50	0.940832
BC	10	7.202452	0.7202451	2.21	0.015641*
ABC	30	15.8452	0.5281735	1.62	0.020041*
S	651	211.8645	0.3254446		
Total (Adjusted)	722	341.0849			
Total	723				

* Term significant at alpha = 0.05

Table 3: Analysis of Variance for D0Ch.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Treatment	3	1027.918	342.6395	5.09	0.001732*
B: Weeks	2	3858.208	1929.104	28.65	0.000000*
AB	6	1714.469	285.7449	4.24	0.000334*
C: Region	5	21513.72	4302.743	63.90	0.000000*
AC	15	356.1189	23.74126	0.35	0.989051
BC	10	483.1798	48.31798	0.72	0.708300
ABC	30	1024.061	34.13536	0.51	0.987566
S	648	43631.5	67.33255		
Total (Adjusted)	719	74852.16			
Total	720				

* Term significant at alpha = 0.05

Table 4: Analysis of Variance for D4Ch

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Treatment	3	16.65017	5.550056	0.89	0.443908
B: Weeks	2	252.0644	126.0322	20.30	0.000000*
AB	6	2.824775	0.4707958	0.08	0.998331
C: Region	5	10169.6	2033.92	327.61	0.000000*
AC	15	61.98639	4.132426	0.67	0.819296
BC	10	47.44838	4.744838	0.76	0.663503
ABC	30	84.65894	2.821965	0.45	0.995009
S	651	4041.68	6.208418		
Total (Adjusted)	722	15068.74			
Total	723				

* Term significant at alpha = 0.05

Table 5: Analysis of Variance for ACh Turnover.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
A: Treatment	3	37.71939	12.57313	1.12	0.340662
B: Weeks	2	172.4933	86.24666	7.68	0.000508*
AB	6	89.44833	14.90806	1.33	0.242832
C: Region	5	14229.96	2845.991	253.27	0.000000*
AC	15	178.4307	11.89538	1.06	0.392496
BC	10	715.3876	71.53876	6.37	0.000000*
ABC	30	297.071	9.902368	0.88	0.651090
S	649	7292.906	11.23714		
Total (Adjusted)	720	23656.05			
Total	721				

* Term significant at alpha = 0.05

A significant effect of treatment was found only for D0Ch, with levels of this variable slightly higher than controls for the PB group, and of weeks post-treatment for D0ACh, D0Ch, D4Ch and ACh Turnover. An interaction of treatment with weeks post-treatment was found for D0ACh, D4 ACh, and D0Ch. There were no significant interactions between treatment and regions for any of the variables.

The mean values of every variable for the different treatments, weeks post-treatment and regions are shown in Figs 7-11. Separate ANOVAs were run for every week post-treatment, shown in Tables 6-8.

Table 6: ANOVAs for 2 weeks post-treatment.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
D0ACh.					
A: Treatment	3	664.2541	221.418	2.93	0.034316*
B: Region	5	82887.65	16577.53	219.34	0.000000*
AB	15	505.2759	33.68506	0.45	0.963714
S	242	18290.57	75.58086		
Total (Adjusted)	265	104028.9			
Total	266				
D4ACh.					
A: Treatment	3	4.811098	1.603699	2.86	0.037543*
B: Region	5	54.92566	10.98513	19.60	0.000000*
AB	15	11.90461	0.7936407	1.42	0.140150
S	242	135.649	0.5605333		
Total (Adjusted)	265	207.1786			
Total	266				
D0Ch.					
A: Treatment	3	1204.716	401.5719	5.83	0.000737*
B: Region	5	8010.301	1602.06	23.25	0.000000*
AB	15	647.623	43.17487	0.63	0.851991
S	242	16677.82	68.91662		
Total (Adjusted)	265	26708.3			
Total	266				
D4Ch.					
A: Treatment	3	4.722415	1.574138	0.31	0.819474
B: Region	5	3382.861	676.5722	132.45	0.000000*
AB	15	61.65257	4.110171	0.80	0.672070
S	242	1236.132	5.107984		
Total (Adjusted)	265	4696.983			
Total	266				
ACh Turnover					
A: Treatment	3	47.9631	15.9877	1.01	0.391218
B: Region	5	6992.311	1398.462	87.91	0.000000*
AB	15	324.1269	21.60846	1.36	0.168810
S	242	3849.648	15.90763		
Total (Adjusted)	265	11325.15			
Total	266				

Table 7: ANOVAs for 4 weeks post-treatment.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
D0ACh.					
A: Treatment	3	624.3097	208.1032	2.04	0.109525
B: Region	5	72128.94	14425.79	141.39	0.000000*
AB	15	618.9841	41.26561	0.40	0.976796
S	203	20712.13	102.0302		
Total (Adjusted)	226	94897.99			
Total	227				
D4ACh.					
A: Treatment	3	1.534196	0.5113985	1.88	0.134425
B: Region	5	33.28047	6.656093	24.45	0.000000*
AB	15	5.805758	0.3870505	1.42	0.139565
S	203	55.27275	0.2722796		
Total (Adjusted)	226	94.98342			
Total	227				
D0Ch.					
A: Treatment	3	1378.589	459.5296	7.10	0.000147*
B: Region	5	7042.813	1408.563	21.76	0.000000*
AB	15	445.2014	29.6801	0.46	0.958258
S	203	13140.95	64.73373		
Total (Adjusted)	226	22231.95			
Total	227				
D4Ch.					
A: Treatment	3	6.619318	2.206439	0.37	0.777331
B: Region	5	3674.576	734.9152	122.04	0.000000*
AB	15	45.41997	3.027998	0.50	0.937385
S	203	1222.445	6.021897		
Total (Adjusted)	226	5022.989			
Total 227					
ACh Turnover.					
A: Treatment	3	62.30194	20.76731	1.68	0.172496
B: Region	5	6145.487	1229.097	99.43	0.000000*
AB	15	124.2458	8.283051	0.67	0.812042
S	202	2496.995	12.36136		
Total (Adjusted)	225	8903.627			
Total 226					

Table 8: ANOVAs for 16 weeks post-treatment.

Source Term	DF	Sum of Squares	Mean Square	F-Ratio	Prob Level
D0ACh.					
A: Treatment	3	431.0621	143.6873	1.14	0.334362
B: Region	5	58858.6	11771.72	93.33	0.000000*
AB	15	820.3923	54.69282	0.43	0.967661
S	200	25226.92	126.1346		
Total (Adjusted)	223	86946.85			
Total	224				
D4ACh.					
A: Treatment	3	0.4979665	0.1659888	1.60	0.189650
B: Region	5	14.90318	2.980636	28.81	0.000000*
AB	15	0.6931203	4.620802E-02	0.45	0.962943
S	200	20.69487	0.1034743		
Total (Adjusted)	223	37.95591			
Total	224				
D0Ch.					
A: Treatment	3	331.0627	110.3542	1.69	0.170386
B: Region	5	6495.704	1299.141	19.90	0.000000*
AB	15	269.6081	17.97387	0.28	0.996921
S	197	12861.6	65.2873		
Total (Adjusted)	220	20407.56			
Total	221				
D4Ch.					
A: Treatment	3	8.208029	2.73601	0.35	0.789439
B: Region	5	3039.041	607.8082	77.68	0.000000*
AB	15	35.44333	2.362889	0.30	0.994864
S	200	1564.984	7.824919		
Total (Adjusted)	223	4872.753			
Total					
ACh Turnover.					
A: Treatment	3	19.8199	6.606633	1.41	0.241398
B: Region	5	2081.042	416.2085	88.75	0.000000*
AB	15	33.54553	2.236369	0.48	0.950182
S	199	933.2097	4.689496		
Total (Adjusted)	222	3121.663			
Total	223				

Multiple contrasts among treatments for the 2 week post-treatment group indicated that D0ACh levels of sarin and sarin+PB differed among them but not from controls. In the case of D4 ACh, PB levels were significantly lower than controls while levels of D0Ch were higher than controls in PB and sarin treated animals.

At four weeks post-treatment, only D0Ch showed significant effects of treatments with higher PB levels than controls in the PB treated animals.

At sixteen weeks post-treatment, none of the variables showed significant effects of treatments.

DISCUSSION AND CONCLUSIONS.

The possible effects of low-dose cholinesterase inhibitors on the function of the autonomic nervous system have significance with regards to clinical findings in a group of veterans from the Persian Gulf (PG) war that complain from a number of symptoms that suggest autonomic dysfunction, such as pathogen-free diarrhea, dizziness and vertigo, night sweats, and sexual dysfunction (Haley et al.,1997). A recent report indicates alterations of circadian variations of heart rate control in a sample of ill PG war veterans (Haley et al.,2004).

Administration of AChE inhibitors at low levels can induce a number changes in the autonomic control of the cardiovascular system. Central AChE inhibition enhances arterial blood pressure (Varagic,1955), (Buccafusco,1996), and decreases cerebrovascular resistance (Scremin and Shih,1991), (Scremin et al.,1993;Scremin et al.,1988). AChE inhibition with pyridostigmine, a carbamate AChE inhibitor that does not cross the blood brain barrier, can induce dose-dependent bradycardia (Stein et al.,1997) or increased arterial blood pressure following a single intravenous dose of 2 mg/kg (Chaney et al.,2002). Enhanced heart rate variability and baroreflex sensitivity during continuous administration with an osmotic minipump of up to 10 mg/kg mass/day have been reported (Bernatova et al.,2003), (Joaquim et al.,2004), with no effects on heart rate or arterial blood pressure. These effects have been reported during the acute administration of ChE inhibitors, but the persistence of these actions beyond the period of drug administration has not been explored to date.

The observation of decreased heart rate two weeks after discontinuation of treatments with PB, sarin or their combination is consistent with residual anticholinesterase effects, since minimal changes were observed at later times after treatment. It is interesting to point out that the bradycardia was observed in the transitional period from night (high activity level) to day (low activity level). On the other hand, the changes in heart rate variability observed at this same time after treatment, took place at the transition from day to night.

Differences in D0ACh, D4ACh, AChT, and D4Ch, among regions reflected the well known variations in cholinergic markers and blood-brain barrier. The concentration of D4Ch in tissue is a function of blood-brain barrier permeability, as well as activity of the high affinity Ch uptake mechanism, and abundance of cholinergic cells in the region. In keeping with this concept, the D4Ch concentration was highest in the infundibulum, one of the few regions within the central nervous system that lacks a blood-brain barrier.

Several effects of treatments were found at 2 weeks, the most persistent being the enhancement of D0Ch levels following PB treatment, that was the only significant change also present at 4 weeks post-treatment. At 16 weeks post-treatment however, no significant changes were detected in any of the variables.

In conclusion, both in the case of heart rate and of all the variables related to cholinergic metabolism, changes were mainly observed 2 weeks after treatment but failed to persist until later intervals. Thus, these experiments do not support the hypothesis that exposure to low levels of cholinesterase inhibitors could induce persistent or delayed effects in heart rate control or cholinergic metabolism.

KEY RESEARCH ACCOMPLISHMENTS DURING THIS PERIOD.

- Heart rate decreased during the transition hours from night to day in animals treated with cholinesterase inhibitors at 2 weeks after discontinuation of treatment. The phenomenon was absent however at later times.
- Heart rate variability, estimated by power spectrum analysis, was enhanced in the early hours of the night period at 2 weeks after treatment with the combination of sarin and PB, but this phenomenon did not persist until later times either.
- Animals showed the expected circadian variation of locomotor activity, with greater levels during the night hours, but the phenomenon was not affected by any of the treatments.
- Several changes in variables of cholinergic metabolism were found at 2 weeks post treatment, but only the elevated D0Ch levels induced by PB treatment could be detected at 4 weeks post-treatment. None of these changes were found 16 weeks after treatment.
- No treatment related effects on ACh turnover were detected at any time after treatment.

REPORTABLE OUTCOMES.

(*) Scremin, O.U., T.M. Shih, L. Huynh, M. Roch, W. Sun, D.R. Chialvo, D.J. Jenden. Low-Dose Cholinesterase Inhibitors Do Not Induce Delayed Effects On Cerebral Blood Flow And Metabolism. *Pharmacology Biochemistry and Behavior (in press, 2005).*

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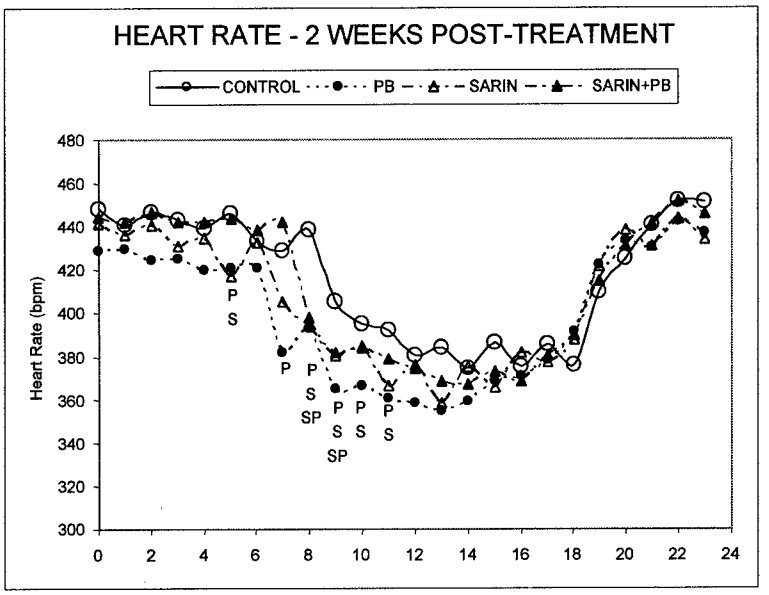


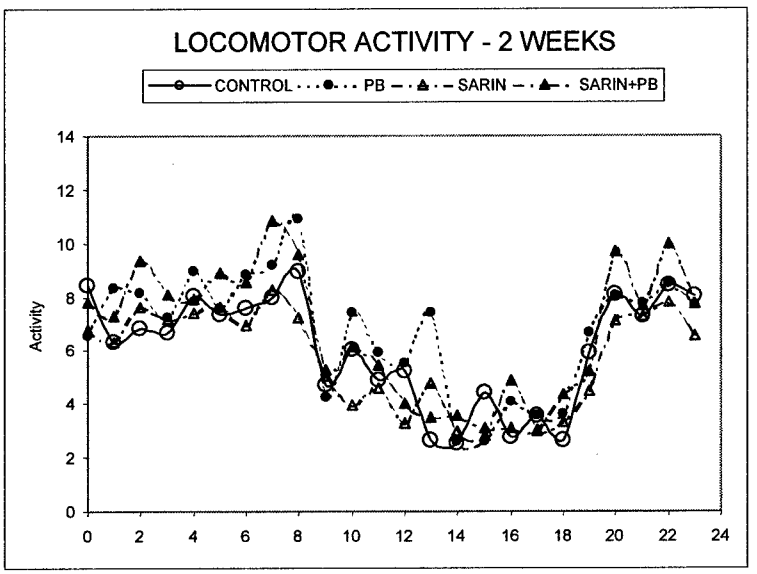
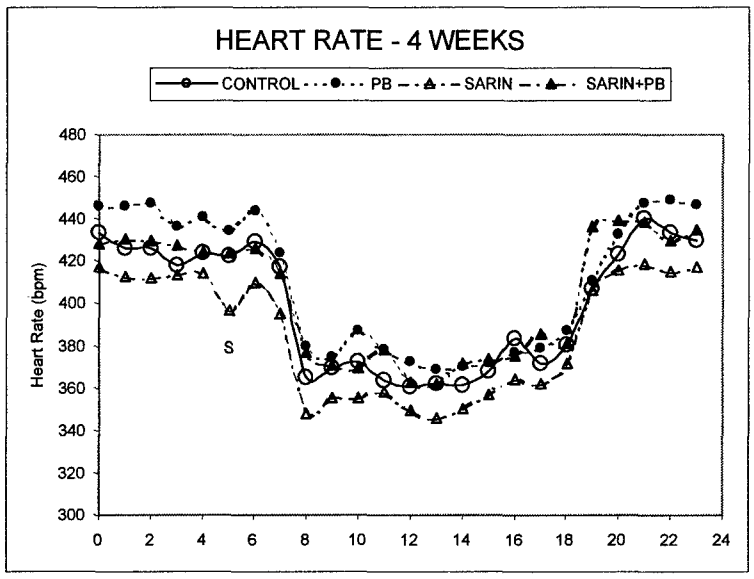


Figure 1: Means of HR (top panel) and LA (bottom panel) as a function of time of day (midnight = 0), measured by telemetry in animals on their home cages during a period of one week, every hour through 24 hrs every day. Measurements were performed during seven days, starting 2 weeks after treatments were discontinued. Means of all days of measurements were averaged for each animal over one hour intervals, and these values in turn were used to calculate group means shown in the graphs. Repeated measures ANOVA was performed at every hour with day of the week as within factor (7 levels), and treatment group as between factor (4 levels). Significant differences from controls (Fisher LSD multiple comparisons procedure, $P<0.05$) are indicated for PB (P) at hours 5, 7, 8, 9, 10, and 11; sarin (S) at hours 5, 8, 9, 10 and 11; and sarin+PB (SP) at hours 8 and 9. No significant differences between controls and treatments were found for LA at any hour. Number of animals: Control=6, PB=7, sarin=6, sarin+PB=7.



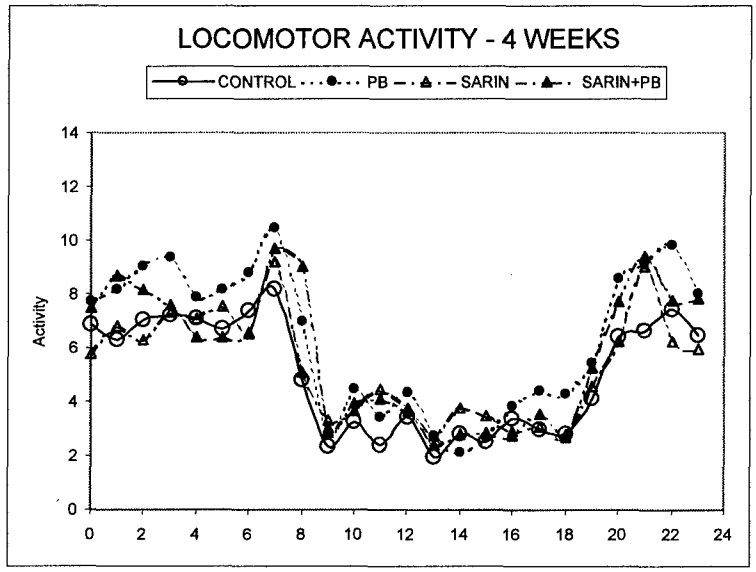
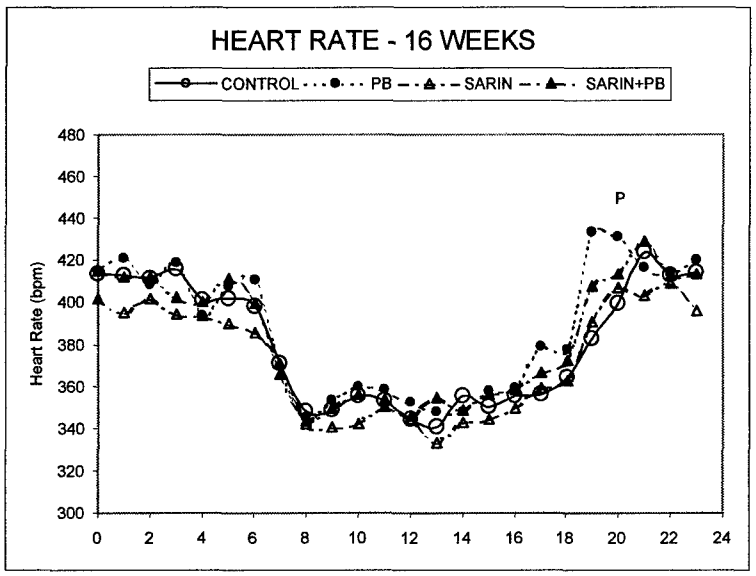


Figure 2: Means of HR (top) panel) and LA (bottom panel) as a function of time of day (midnight = 0), measured by telemetry in animals on their home cages during a period of one week, every hour through 24 hrs every day. Measurements were performed during seven days, starting 4 weeks after treatments were discontinued. Means of all days of measurements were averaged for each animal over one hour intervals, and these values in turn were used to calculate group means shown in the graphs. Repeated measures ANOVA was performed at every hour with day of the week as within factor (7 levels), and treatment group as between factor (4 levels). Significant difference from controls (Fisher LSD multiple comparisons procedure, $P<0.05$) is indicated for sarin (S) at hour 3. No significant differences between controls and treatments were found for LA at any hour. Number of animals: Control=8, PB=8, sarin=8, sarin+PB=6.



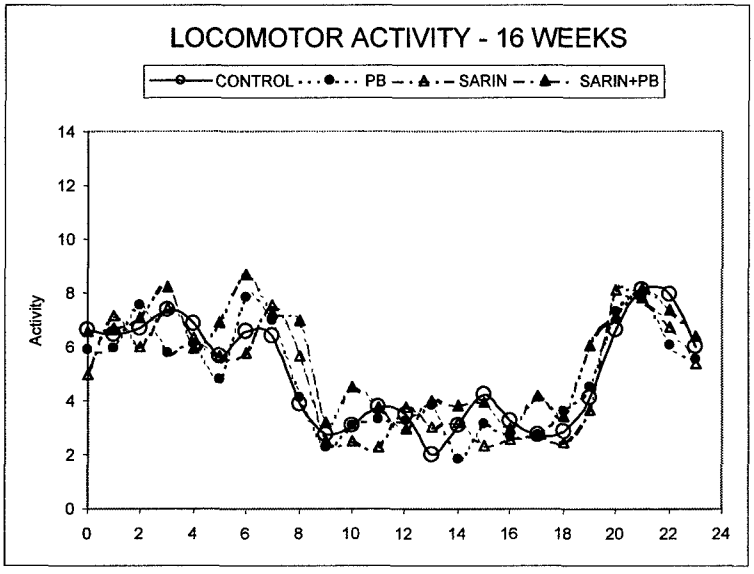


Figure 3: Means of HR (top panel) and LA (bottom panel) as a function of time of day (midnight = 0), measured by telemetry in animals on their home cages during a period of one week, every 30 minutes, 24 hrs every day. Measurements were performed during seven days, starting 16 weeks after treatments were discontinued. Means of all days of measurements were averaged for each animal over one hour intervals, and these values in turn were used to calculate group means shown in the graphs. Repeated measures ANOVA was performed at every hour with day of the week as within factor (7 levels), and treatment group as between factor (4 levels). Significant difference from controls (Fisher LSD multiple comparisons procedure, $P<0.05$) is indicated for PB (P) at hour 20. No significant differences between controls and treatments were found for LA at any hour. Number of animals: Control=8, PB=8, sarin=8, sarin+PB=6

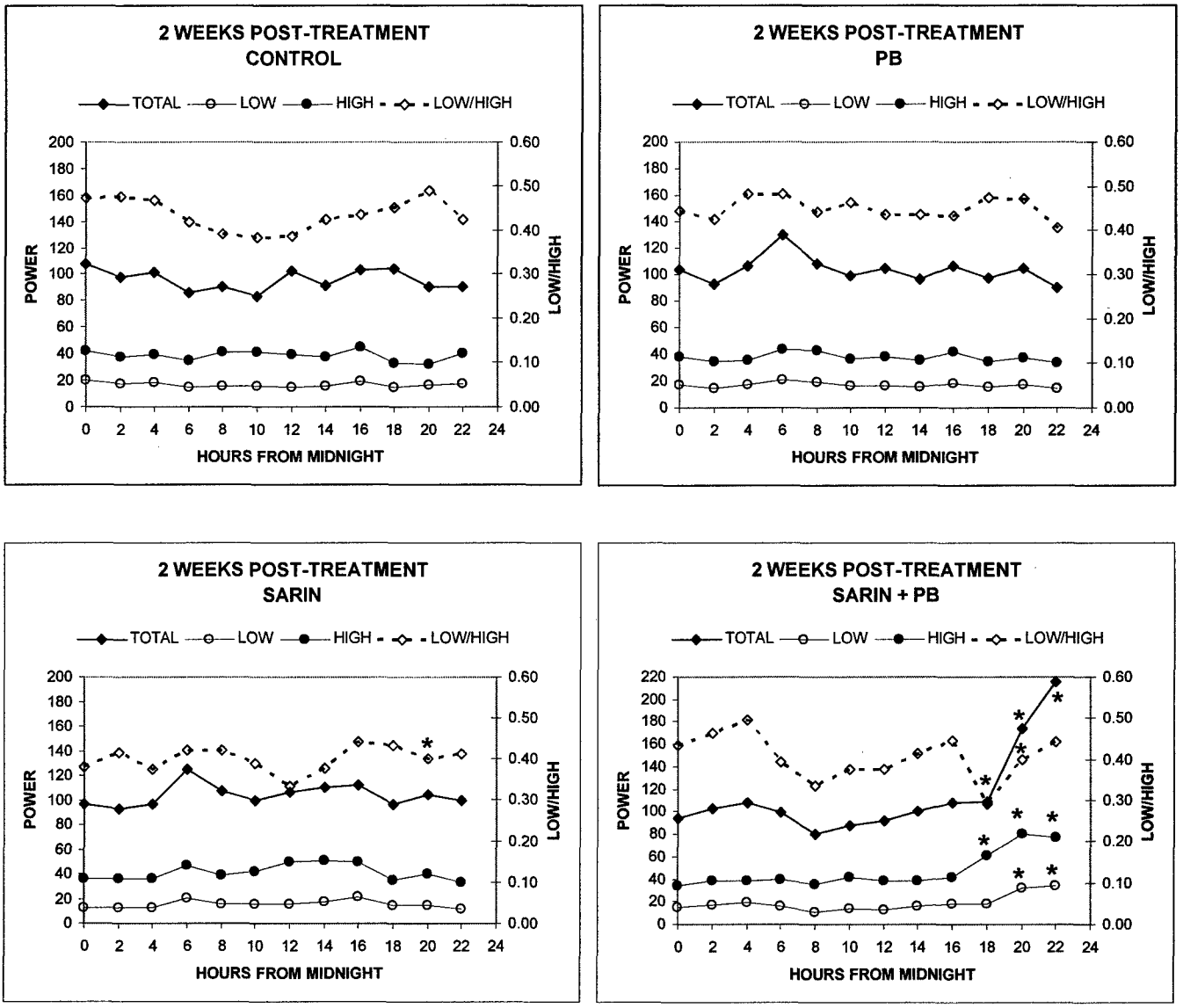


Figure 4: Means of HR cumulative power over the following spectrum frequency bands: TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz), HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 minutes by radiotelemetry during seven days. Means of the third day of measurements were averaged for each animal over two hour intervals, and these values in turn were used to calculate group means. Data shown corresponds to animals studied 2 weeks after discontinuation of treatment with PB (top right, number of animals (n) =7), sarin (bottom left, n=6) and sarin + PB (bottom right, n=7). Statistical significance ($P<0.05$) of differences from the control group (top left, n=6) are indicated by an asterisk.

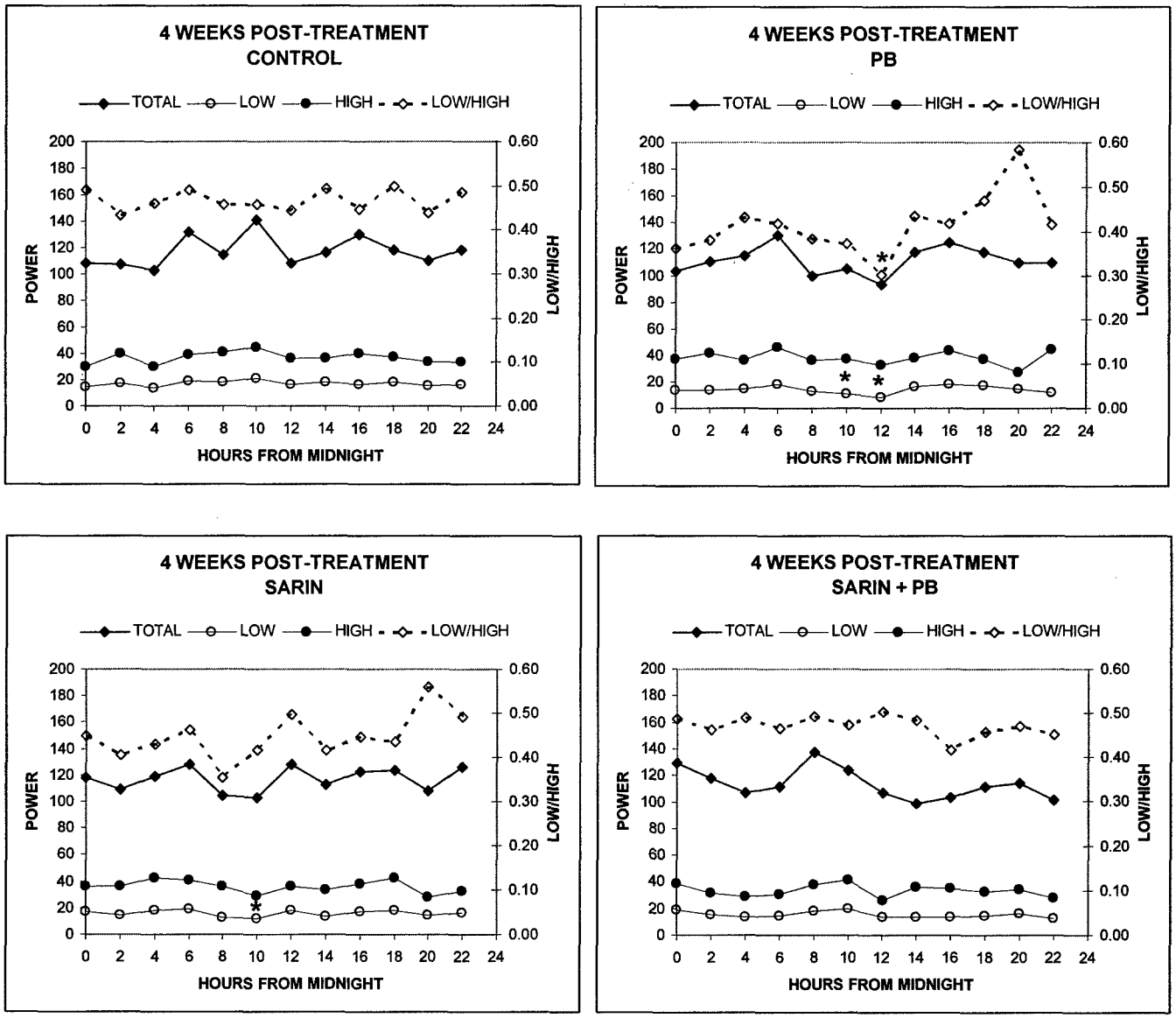


Figure 5: Means of HR cumulative power over the following spectrum frequency bands: TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz), HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 minutes by radiotelemetry during seven days. Means of the third day of measurements were averaged for each animal over two hour intervals, and these values in turn were used to calculate group means. Data shown corresponds to animals studied 4 weeks after discontinuation of treatment with PB (top right, number of animals (n) =8), sarin (bottom left, n=8) and sarin + PB (bottom right, n=6). Statistical significance ($P<0.05$) of differences from the control group (top left, n=8) are indicated by an asterisk.

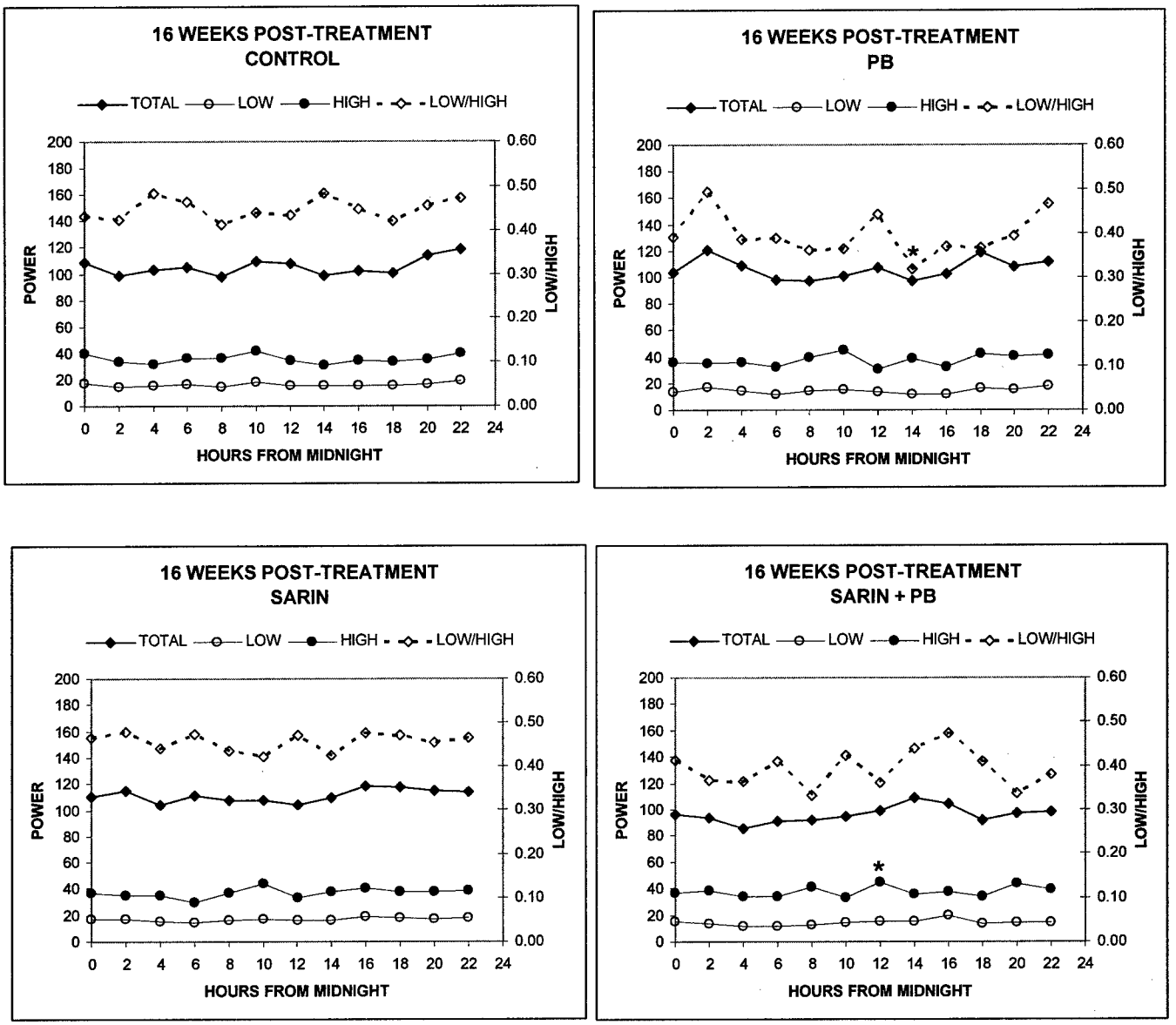
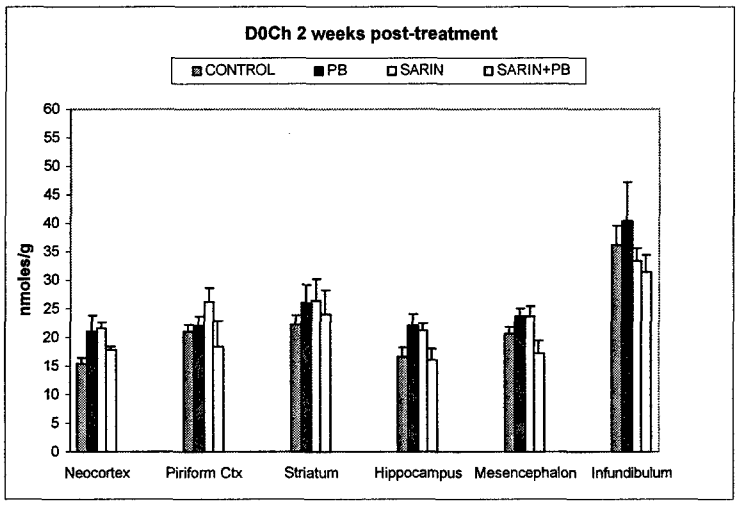
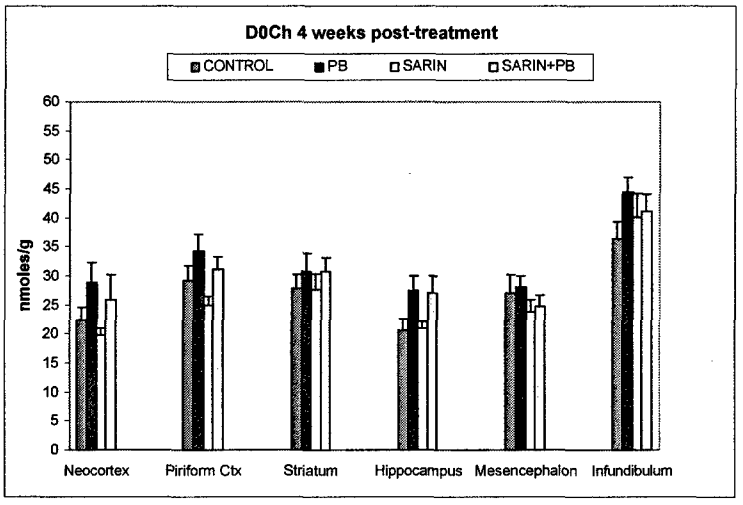


Figure 6: Means of HR cumulative power over the following spectrum frequency bands: TOTAL (between 0.05 and 5 Hz), LOW (between 0.26 and 0.75 Hz), HIGH (between 0.76 and 5 Hz), and the ratio of power in low to high frequency bands (LOW/HIGH). HR was calculated from the electrocardiogram recorded every 30 minutes by radiotelemetry during seven days. Means of the third day of measurements were averaged for each animal over two hour intervals, and these values in turn were used to calculate group means. Data shown corresponds to animals studied 16 weeks after discontinuation of treatment with PB (top right, number of animals (n) =8), sarin (bottom left, n=6) and sarin + PB (bottom right, n=7).. Statistical significance ($P<0.05$) of differences from the control group (top left, n=7) are indicated by an asterisk.





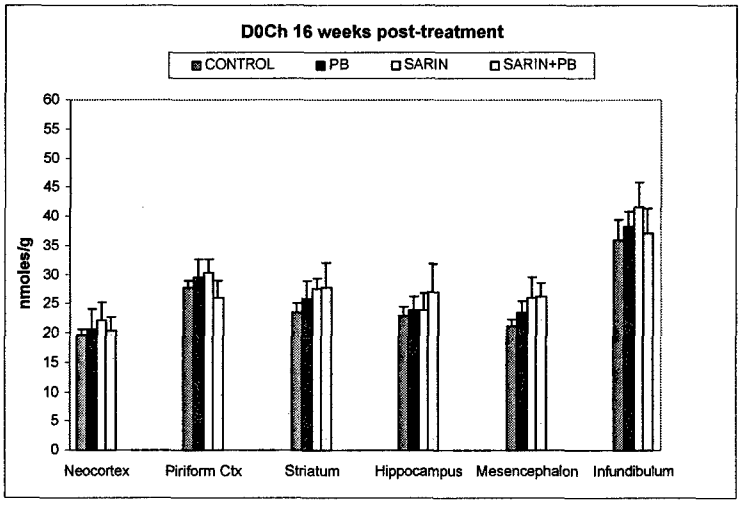


Figure 7: Mean and SE of tissue D0Ch, for all regions sampled 2 weeks (top panel), 4 weeks (middle panel), and 16 weeks (bottom panel) after treatment. Statistics described in text and Tables 6-8.

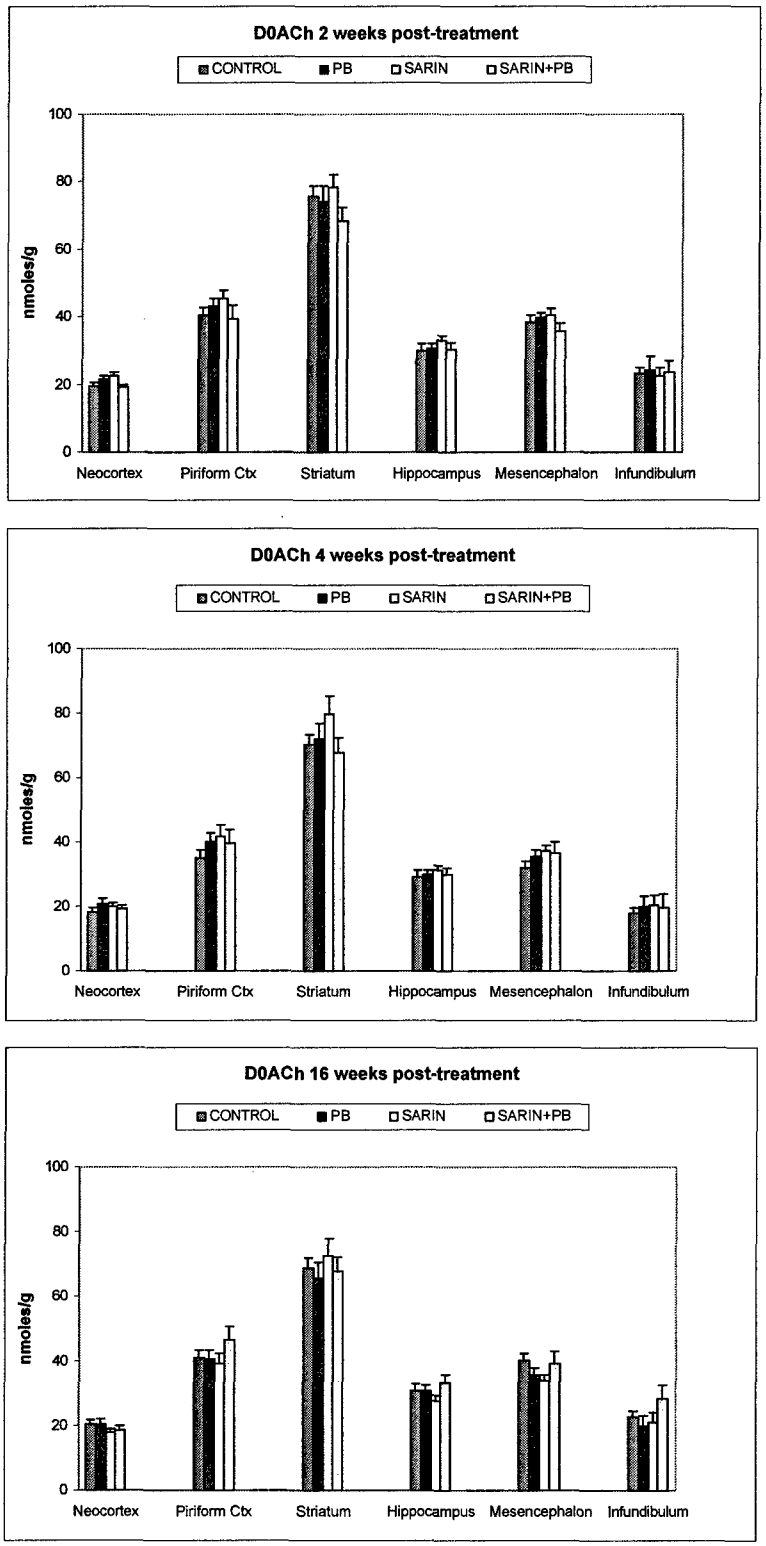


Figure 8: Mean and SE of tissue D0ACh, for all regions sampled 2 weeks (top panel), 4 weeks (middle panel), and 16 weeks (bottom panel) after treatment. Statistics described in text and Tables 6-8.

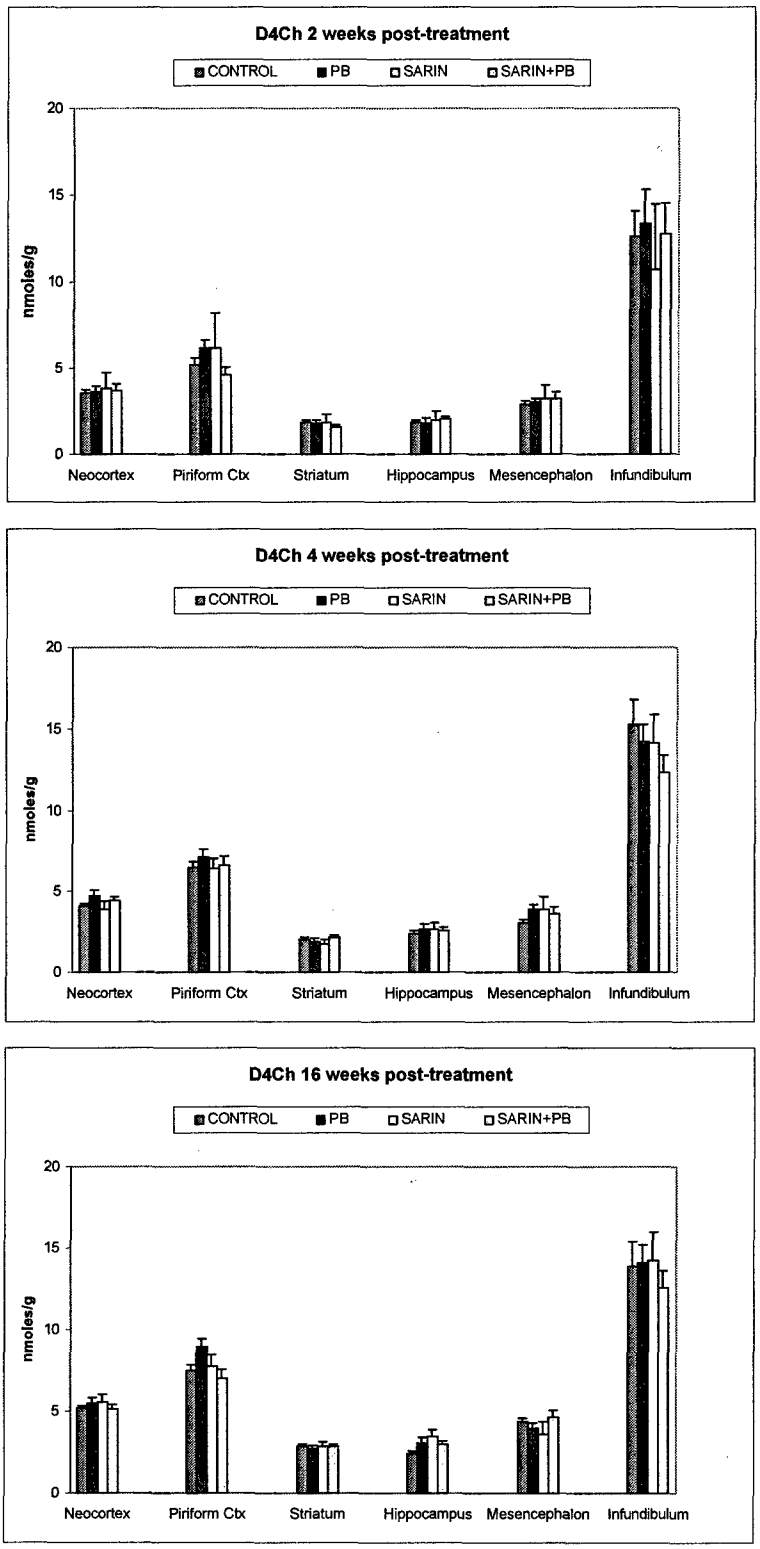


Figure 9: Mean and SE of tissue D4Ch, for all regions sampled 2 weeks (top panel), 4 weeks (middle panel), and 16 weeks (bottom panel) after treatment. Statistics described in text and Tables 6-8.

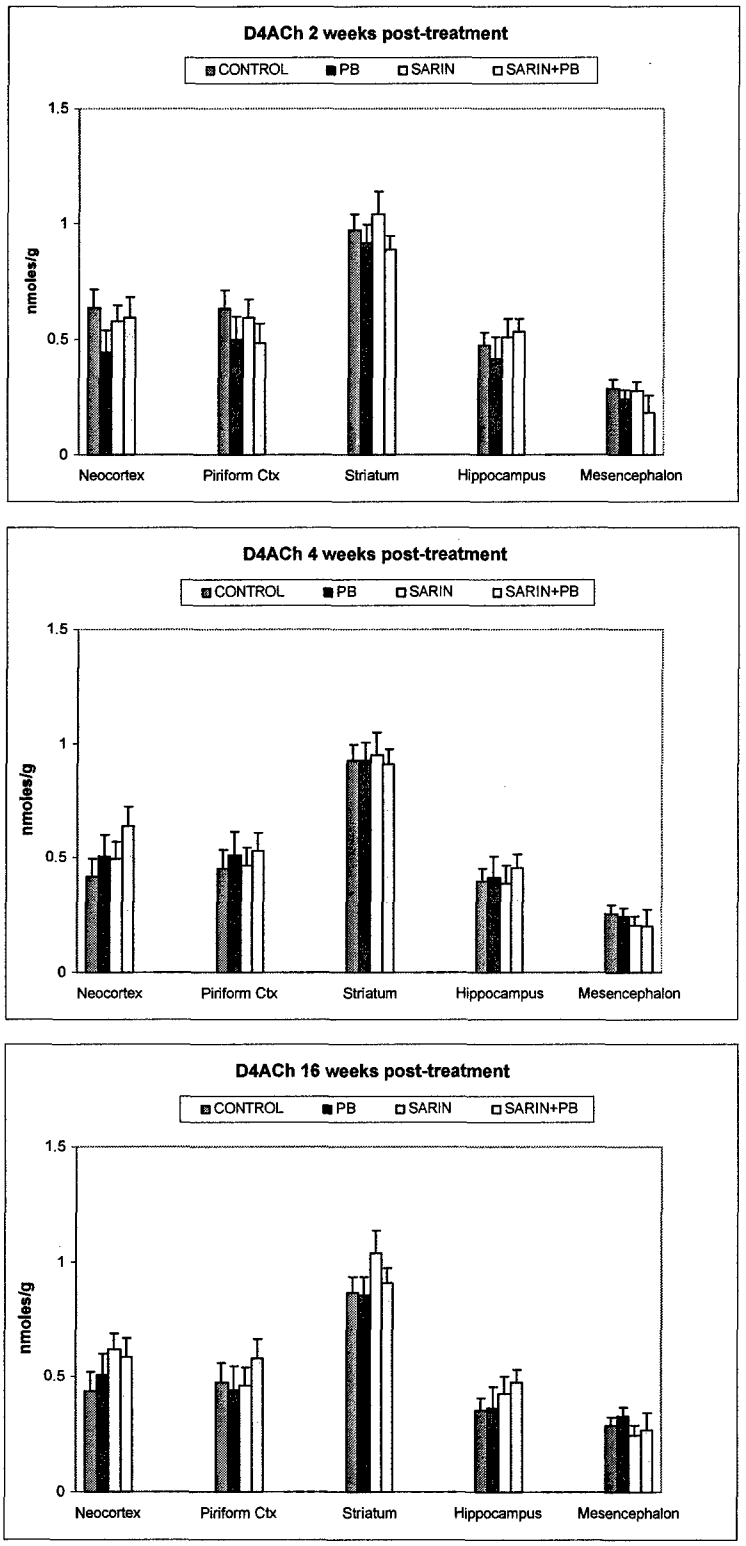


Figure 10: Mean and SE of tissue D4ACh, for all regions sampled 2 weeks (top panel), 4 weeks (middle panel), and 16 weeks (bottom panel) after treatment. Statistics described in text and Tables 6-8.

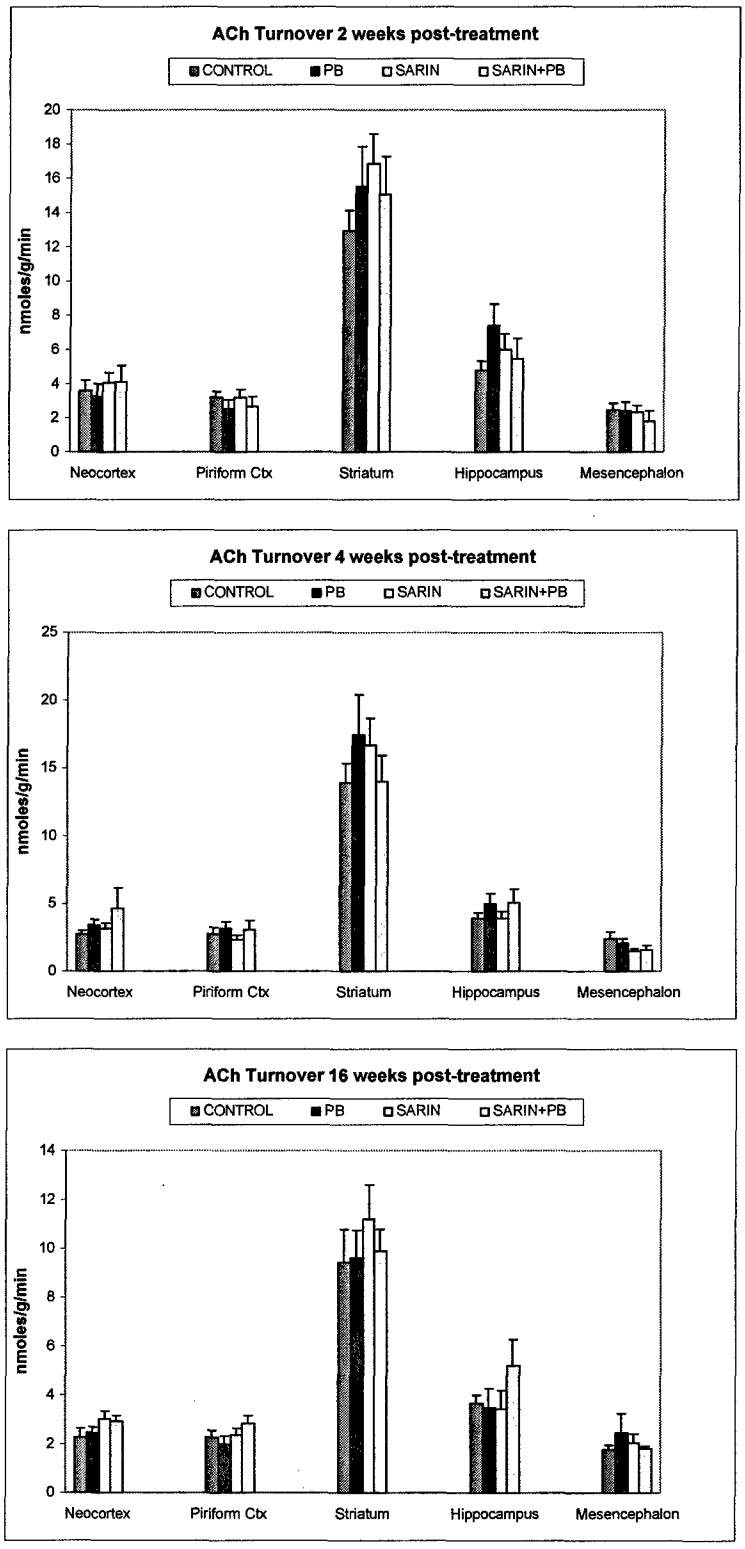


Figure 11: Mean and SE of tissue ACh Turnover, for all regions sampled 2 weeks (top panel), 4 weeks (middle panel), and 16 weeks (bottom panel) after treatment. Statistics described in text and Tables 6-8.

APPENDIX.

Article in press

LOW-DOSE CHOLINESTERASE INHIBITORS DO NOT INDUCE DELAYED EFFECTS ON CEREBRAL BLOOD FLOW AND METABOLISM

Oscar U. Scremin, Tsung-Ming Shih, Ly Huynh, Margareth Roch, Wei Sun, Dante R. Chialvo, Donald J. Jenden.

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Low-dose cholinesterase inhibitors do not induce delayed effects on cerebral blood flow and metabolism

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Abstract

The acetylcholinesterase (AChE) inhibitors sarin and pyridostigmine bromide (PB) have been proposed as causes of neurobehavioral dysfunction in Persian Gulf War veterans. To test possible delayed effects of these agents, we exposed rats to low (subsymptomatic) levels of sarin (0.5 LD50 s.c. 3 times weekly) and/or PB (80 mg/L in drinking water) for 3 weeks. Controls received saline s.c. and tap water. At 2, 4 and 16 weeks after exposure, regional cerebral blood flow (rCBF) and glucose utilization (rCGU) were measured in conscious animals with the Iodo-^{14}C-antipyrine and ^{14}C-2 deoxyglucose methods, respectively.

Two weeks after exposure, PB+sarin caused significant rCBF elevations, but no changes in rCGU, in neocortex, with lesser effects on allocortex. Four weeks after exposure, the same general pattern was found with sarin. Only a few changes were found at 16 weeks post-treatment. The predominant effects of sarin or PB+sarin on rCBF at earlier times after treatment are consistent with the well known direct cerebral vascular effect of cholinergic agonists. The lack of changes in rCBF and rCGU observed at 16 weeks after treatment does not support the hypothesis that repeat exposure to low-dose cholinesterase inhibitors can generate permanent alterations in cerebral activity.

Keywords: Nerve agents; Sarin; Pyridostigmine bromide; Cerebral glucose utilization; Cerebrovascular circulation; Low dose cholinesterase toxicity

1. Introduction

Although the immediate and long term consequences of acute intoxication with organophosphorus (OP) AChE inhibitors are well known (Ecobichon and Joy, 1982; Sidell, 1974; Chambers, 1992), potential harmful delayed effects of repeated exposure to low (non-symptomatic) levels of these agents have attracted less attention. These effects may be of relevance, if they exist, to military personnel possibly exposed to non-symptomatic levels of sarin during the Persian Gulf War (McCauley et al., 2001), and to agricultural workers and the general population exposed to OP insecticides of widespread use.

Administration of AChE at low levels generates a number of physiological changes. Central AChE inhibition

Abbreviations: AChE, Acetylcholinesterase; BuChE, Butyrylcholinesterase; ChAT, Cholineacetyltransferase; DG, Deoxyglucose; IAP, Iodo-antipyrine; OP, Organophosphorus; PB, Pyridostigmine bromide; RBC, Red blood cells; rCBF, Regional cerebral blood flow; rCGU, Regional cerebral glucose utilization; Am, Amygdala; Au, Auditory; Au1, Primary auditory; BF, Barrel field; Ect, Ectorhinal; Ent, Entorhinal; Fa, Face area; FL, Forelimb area; HL, Hindlimb area; I, Insular; M1, Primary motor; M2, Secondary motor; PA, Parietal association area; Pir, Piriform; RS, Retrosplenial; S1, Primary somatosensory; S2, Secondary somatosensory; Te, Temporal; Tr, Trunk area; V1, Primary visual; V2, Secondary visual.

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enhances arterial blood pressure (Varagic, 1955; Buccafusco, 1996), and decreases cerebrovascular resistance (Scremin and Shih, 1991; Scremin et al., 1993; Scremin et al., 1988), decreases body temperature and elevates nociceptive threshold (Russell et al., 1986). AChE inhibition with pyridostigmine, a carbamate AChE inhibitor that does not cross the blood brain barrier, can induce dose-dependent bradycardia (Stein et al., 1997) or increased arterial blood pressure following a single intravenous dose of 2 mg/kg (Chaney et al., 2002) but no effects on arterial blood pressure or heart rate but enhanced heart rate variability and baroreflex sensitivity during continuous administration with an osmotic minipump of up to 10 mg/kg mass/day (Bernatova et al., 2003; Joaquim et al., 2004). However, The persistence of these effects beyond the period of drug administration has not been explored.

Previous work on delayed effects of low dose OP AChE inhibition has led to contradictory results. While some authors have reported some effects (Burchfield and Duffy, 1982; Ecobichon and Joy, 1982), other studies have found no increase over the general population in the incidence of mental, neurological, hepatic, and reproductive pathology or cancer of subjects exposed in the work environment (Panel on Anticholinesterase Chemicals, 1982) or after accidental exposures (Coordinating Subcommittee, 1985; Moore, 1998).

The present study was designed to determine whether exposure to sarin and/or PB, in doses and times that presumably applied to Persian Gulf War veterans, could elicit long lasting alterations in the patterns of cerebral cortex activity of conscious animals, as assessed with measurements of cerebral blood flow and metabolism, and thus test whether the agents could elicit subtle delayed effects of cerebral function. Quantitative autoradiography with ^{14}C-labeled Iodo-antipyrine (IAP) and 2-deoxyglucose (2-DG) was used to trace regional cerebral blood flow (rCBF) and regional cerebral glucose utilization (rCGU) of the cerebral cortex, respectively. Spatial resolution of these techniques is on the order of 10 lines/mm (Gallistel and Nichols, 1983), allowing identification of the small, specialized areas of the rat cerebral cortex. These variables have been extensively used to reveal patterns of activity in the central nervous system (McCulloch, 1982; Sokoloff, 1981; Reivich et al., 1969; Sakurada et al., 1978; Holschneider et al., 2003). The somatosensory, auditory, motor, visual and association areas of the neocortex, as well as the various components of the allocortex were sampled in the treated animals and compared with age-matched controls.

Comparison of rCBF and rCGU on a regional basis is particularly suited to the present study. One well documented acute effect of AChE inhibitors, like sarin, that penetrate the blood–brain barrier is an increase in rCBF predominantly in the neocortex without a concomitant increase in rCGU at low (non-symptomatic) doses (Scremin et al., 1988; Scremin and Shih, 1991). A generalized increase in both variables was observed at convulsant doses (Shih and Scremin, 1992). Thus, the rCBF/rCGU ratio provides a sensitive indicator of the extent and intensity of the central nervous system effects of AChE inhibitors.

This study also includes PB exposure, because this agent has been used as a pretreatment for nerve agent intoxication during the Persian Gulf War (Keeler et al., 1991), and there are also contradictory reports on the potential long-term undesirable effects of exposure to PB, with some authors reporting a delayed enhancement of the acoustic startle response in rats (Servatius et al., 1998) and others showing no significant effects on this variable (Scremin et al., 2003).

The experimental model used was developed previously for the evaluation of neurologic and cognitive delayed effects of sarin and PB (Scremin et al., 2003). It consists of PB administration in the drinking water at a concentration of 80 mg/ml for 3 weeks, in combination with sarin ($0.5\times LD_{50}$, s.c.; three injections per week for 3 weeks) or an equivalent volume of saline s.c. at the same regime. These treatments induced no signs of intoxication, even though red blood cell AChE was inhibited between 60% and 70%.

2. Materials and methods

Male Crl:CD(SD)IGSBR Sprague–Dawley rats, weighing 250–300 g at the beginning of treatment, were used in these studies. Animals were obtained from Charles River Labs (Kingston, NY) and housed individually in temperature (21±2 °C) and humidity (50±10%) controlled animal quarters maintained on a 12-h light–dark full spectrum lighting cycle with lights on at 0600 hours. Laboratory chow and drinking water were freely available.

Experiments were conducted at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD) and the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System. The research environment and protocols for animal experimentation were approved at each site by their respective institutional animal care and use committees. Animal facilities at both institutions are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The animals used in these studies were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals, proposed by the Committee to Revise the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and published by National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as reflecting the views of the Department of the Army or the Department of Defense.

Saline (0.9% NaCl) injection, USP, was purchased from Cutter Labs. (Berkeley, CA). Sarin, obtained from the U.S.

Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD), was diluted in ice-cold saline prior to injection. Saline or sarin injection volume was 0.5 mL/kg subcutaneously (s.c.). PB was purchased from Sigma-Aldrich (St. Louis, MO) and prepared twice weekly in tap water and provided as drinking water to experimental groups for a 3-week period.

2.1. Experimental groups

Separate sets of animals were studied at 2, 4, or 16 weeks after treatment. Within every set, animals were divided into four treatment groups. Group 1 served as overall control. These animals received regular tap water as drinking water and were injected with saline (Control group). Group 2 animals received PB in drinking water (80 mg/L), with an estimated dose, based on water consumption, of 10 mg/kg body mass/day (Scremin et al., 2003), approximately equivalent to the dose used in humans for prophylaxis of OP poisoning (1.2 mg/kg body mass/day), based on surface area dosage conversion between species (Freireich et al., 1966). These animals (PB group) were injected with saline s.c. Group 3 animals received tap water and were injected with sarin (62.5 μg/kg, s.c., equivalent to $0.5 \times LD_{50}$) (sarin group). Group 4 rats received PB in drinking water and were injected with sarin at the doses stated above (PB+sarin group). PB in drinking water was provided continuously to animals in groups 2 and 4, starting on Monday morning at 0800 hours. At 0900 hours that Monday morning, injection of either saline (0.5 mL/kg, s.c.) or sarin (62.5 μg/kg, s.c.) was initiated. The injection was given three times (Mondays, Wednesdays, and Fridays) per week. PB in drinking water was terminated and switched to regular tap water at 1700 hours on Friday of the third week. Animal dosing procedures were performed at the USAMRICD laboratory. After a period of 1, 3, or 15 weeks following treatment, depending on the experimental sets, animals were transported by air-conditioned vans and air-freight to the Laboratory of Neurophysiology, VA Greater Los Angeles Healthcare System, where they were allowed to recover for a minimum of one additional week before starting assessment of the outcome variables at 2, 4, or 16 weeks after control, PB, sarin, or PB+sarin treatments. Telemetry measurements of locomotor activity and heart rate performed in animals after they arrived at the VA Greater Los Angeles Healthcare System (data not shown), have indicated normal circadian rhythms in animals transported under the same conditions and studied at the intervals used in the present report. Moreover, in this experimental design all animals (treated and controls in each experimental set) were transported in the same way at the same time to control for any potential differences due to transportation stress.

The 12 groups of animals described above (4 treatments×3 times after treatment) were duplicated to measure rCBF and rCGU, since only one of these methodologies was used on a single animal.

2.2. Observation of signs of intoxication

Animals were observed for signs of cholinergic intoxication for at least 1 h following sarin injection. The signs, including motor dysfunction (fasciculations, tremors, convulsions), gland secretion (salivation, lacrimation), eye bulb protrusion, and general state (activity and coordination) were scored according to the rating schedule described elsewhere (Shih and Romano, 1988).

2.3. Blood AChE measurements

When animals were received at the USAMRICD laboratory, they were allowed to acclimate for a week. During this period blood was collected from the tail vein (Liu et al., 1999) on 2 separate days to establish baseline whole blood and red blood cell (RBC) AChE activity. After the experiment was started on the following Monday, subsequent blood collections were done on each Friday, at about 60 min after sarin or saline injections, during the 3-week exposure period and continued for 3 more weeks during the recovery period. Blood (0.25 mL) was collected into an Eppendorf 1.5 mL microtube containing 50 μL (1000 USP unit per mL) heparin sodium and mixed. Forty microliters of whole blood was transferred to another microtube containing 160 μL 1% Triton-X 100 (in saline) solution, mixed well and immediately flash frozen. The remaining blood was then centrifuged for 5 min at 14,000 RPM (20,000 RCF). Plasma was carefully aspirated off, and 20 μL RBC's was transferred into a microtube containing 180 μL 1% Triton-X 100 solution. The tube was tapped firmly until RBC's were lysed and dispersed. The tube was immediately flash frozen. Both the whole blood and RBC samples were stored at −75 °C until ChE analysis. At the time of analysis, samples were processed immediately after thawing to avoid spontaneous re-activation or additional inhibition of ChE activity. Whole blood and RBC AChE activity were determined by an automated method using a COBAS/FARA clinical chemistry analyzer (Roche Diagnostics, Nutley, NJ). The analytical procedure was based on the manual method of Ellman (Ellman et al., 1961) and modified for the COBAS/FARA system using acetylthiocholine as substrate.

2.4. Measurement of cerebral glucose utilization

Regional cerebral glucose utilization (rCGU) was measured with the ^{14}C 2-DG autoradiographic technique (Sokoloff et al., 1977). One arterial and one venous catheter were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for 1 h. In these cages the animals rest in prone position with their limbs hanging to the sides. Acrylic non-traumatic bars entrap the animal preventing locomotion but allowing limb and head movements. The cage was covered with a cloth in order to

prevent cooling of the animal and to eliminate visual contact with the environment. Rectal temperature was recorded with a BAT-12 thermocouple thermometer and maintained constant by means of a TCAT-1A (Physitemp, Clifton, NJ) temperature controller and a source of radiant heat. A sample of arterial blood was obtained for measurement of blood gases and pH in an ABL-5 blood acid-base system (Radiometer, Copenhagen, Denmark) and then ^{14}C 2-DG (Amersham, Arlington Heights, IL) dissolved in 0.5 mL of saline at a concentration of 100 μCi/kg body mass was administered intravenously at a rate of 1 mL/min for 30 s. Eleven arterial blood samples (70 μL) were then obtained over a period of 45 min for measurement of glucose concentration (glucose oxidase method) and radioactivity (liquid scintillation counting) to allow calculation of rCGU. After euthanasia (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus), performed immediately after obtaining the last blood sample (45 min after ^{14}C 2-DG infusion) the brain was removed, flash frozen in methylbutane chilled to −70 °C and embedded in OCT compound (Miles, Elkhart, Indiana) for later sectioning in a cryostat at −20 °C in 20 μm slices. These sections were heat-dried and exposed to Kodak Ektascan film in spring-loaded X-ray cassettes along with eight standards of known radioactivity to obtain a ^{14}C-2-DG autoradiograph. Tissue radioactivity was derived by densitometry of tissue and standards autoradiographs and rCGU values were obtained using the operational equation and values for the lumped and rate constants previously described (Sokoloff et al., 1977).

2.5. Measurement of cerebral blood flow

Regional cerebral blood flow (rCBF) was measured with the ^{14}C-IAP quantitative autoradiographic method (Sakurada et al., 1978). Two arterial and two venous catheters were implanted in the femoral vessels under halothane anesthesia. After surgery, animals were placed in a Bollman cage and allowed to recover from anesthesia for 1 h. Rectal temperature was recorded with a BAT-12 thermocouple thermometer connected to a TCAT-1A (Physitemp) temperature controller and a source of radiant heat. One arterial catheter was connected to a pressure transducer interfaced to a polygraph for continuous recording of arterial blood pressure; the other one was used for sampling of arterial blood. One of the venous catheters was connected to a motor driven syringe containing the radioactive tracer solution and the other one to a similar syringe containing the euthanasia solution (pentobarbital, 50 mg/kg with 3 M KCl i.v. bolus). A sample of arterial blood was obtained for measurement of blood gases and pH in a Radiometer ABL-5 blood acid-base system and then the infusion of ^{14}C-IAP (Amersham, Arlington Heights, IL) was started. Infusate volume was 0.6 mL, dose was 100 μCi/kg and infusion period was 30 s. Arterial blood samples (30 μL) were obtained every 3 s from a free flowing catheter. Circulation was arrested by the euthanasia solution delivered intravenously over the last 4 s of the ^{14}C-IAP infusion. The exact timing of circulatory arrest was determined from the polygraph record of arterial blood pressure. The brain was then rapidly removed and processed for autoradiography as described above for rCGU measurements. rCBF was calculated from film optical density of brain autoradiographs and standards, and arterial blood radioactivity as described previously (Sakurada et al., 1978).

Although pentobarbital was used in these experiments, the drug cannot affect rCBF or rCGU because it is administered after the uptake of the blood flow or metabolism tracers has taken place and after the last sample of blood has been collected (during the last 4 s of the rCBF experiments or after the last blood sample of the rCGU experiment).

2.6. Cerebral cortical regions sampled

The following regions, identified according to the Atlas of Paxinos and Watson (Paxinos and Watson, 1998) were sampled for measurements of rCBF and rCGU in 20 locations in each of 15 coronal planes spaced 0.4 mm from each other. The numbers of locations per region sampled in every animal are indicated in the following list after the abbreviation: Neocortex: auditory cortex (Au, 4), primary auditory cortex (Au1, 8), barrel cortical field (BF, 16), face cortical area (Fa, 26), forelimb cortical area (FL, 10), hindlimb cortical area (HL, 6), insular cortex (I, 22), primary motor cortex (M1, 22), secondary motor cortex (M2, 18), parietal association area (PA, 4), primary somatosensory cortex (S1, 2), secondary somatosensory cortex (S2, 8), temporal cortex (Te, 12), trunk cortical area (Tr, 4), primary visual cortex (V1, 20), and secondary visual cortex (V2, 20); allocortex and transitional areas: ectorhinal cortex (Ect, 6), entorhinal cortex (Ent, 24), piriform cortex (Pir, 48), retrosplenial cortex (RS, 12); amygdala (Am, 8).

2.7. Data analysis

Means of AChE activity for every treatment group were calculated. Statistical significance of differences between every treatment group and the respective controls at each time after treatment was assessed by analysis of variance (ANOVA) followed by multiple comparisons with the Bonferroni technique (Snedecor and Cochran, 1980). A probability of <0.05 (<0.016 after Bonferroni adjustment) was used to declare differences as significant. Mean values of rCBF and rCGU were calculated for every location sampled in all experimental groups and times after treatment. Statistical significance of location means of drug treatment groups against those of their respective controls at each time after treatment was assessed by multiple comparisons with the Bonferroni technique as described above.

The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental

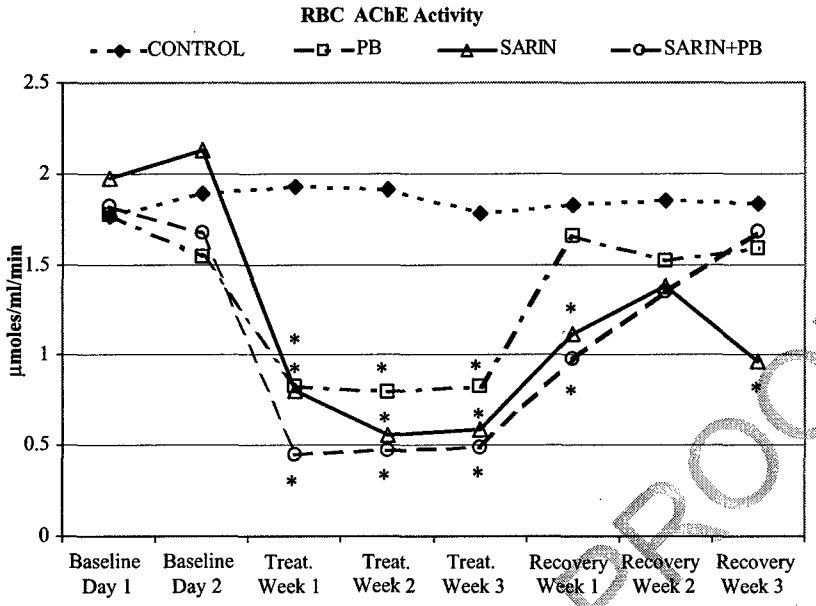

Fig. 1. RBC AChE activity was measured before (baseline), during treatment (treatment weeks 1–3) and the immediate recovery period (recovery weeks 1–3). Data (Means and SE) are in µmol/mL/min. *=significant vs. controls ($P<0.05$, Bonferroni adjusted for three contrasts).

group. Statistical significance of differences between slopes of the three drug treated groups against their respective controls for every time after treatment was assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted (Snedecor and Cochran, 1980). The significance level was set at 0.01 to compensate for the multiple comparisons performed.

2.8. Number of animals

rCBF groups, 2 weeks after treatment: Control=12; PB=10; Sarin=12; PB+Sarin=10; 4 weeks after treatment: Control=11; PB=8; Sarin=11; PB+Sarin=10; 16 weeks after treatment: Control=11; PB=7; Sarin=8; PB+Sarin=11. Number of animals in rCGU groups, 2 weeks after treatment: Control=9; PB=6; Sarin=5; PB+Sarin=8; 4 weeks after treatment: Control=7; PB=7; Sarin=8; PB+Sarin=8; 16 weeks after treatment: Control=5; PB=6; Sarin=7; PB+Sarin=5.

3. Results

3.1. Blood cholinesterase activity

Measurements of RBC AChE during drug treatment and the immediate recovery period are shown in Fig. 1. PB induced a pronounced decrease in enzymatic activity to

Table 1
Physiological variables in all rCBF and rCGU experiments

Treat.	Weeks	Blood pH-log [H^+]	Pa CO_2 (mmHg)	PaO_2 (mmHg)	Body mass (g)	Body temp (°C)	MABP (mm Hg)
Control	2	7.453±0.003	40.80±0.57	86.62±1.15	448.1±8.6	37.7±0.1	118.0±4.1
PB	2	7.456±0.008	41.15±0.86	85.08±1.36	455.6±9.5	38.0±0.1	110.2±3.4
Sarin	2	7.446±0.006	40.11±0.60	84.33±1.68	464.9±8.6	37.8±0.1	122.5±2.8
Sarin+PB	2	7.461±0.007	40.43±0.80	87.36±1.14	454.8±8.0	37.9±0.1	120.4±3.2
Control	4	7.452±0.004	40.28±0.80	85.89±1.27	482.5±10.6	37.7±0.1	119.5±2.4
PB	4	7.448±0.006	41.03±0.68	86.36±0.79	510.3±10.8	37.9±0.2	121.4±6.4
Sarin	4	7.446±0.004	39.00±0.57	84.63±0.94	483.9±10.0	37.8±0.2	117.5±3.8
Sarin+PB	4	7.451±0.004	40.24±0.49	89.19±1.41	491.4±11.1	37.8±0.1	126.2±3.3
Control	16	7.441±0.005	40.50±0.57	86.75±1.20	609.8±10.4	37.6±0.1	110.6±4.0
PB	16	7.437±0.005	40.61±0.52	84.36±1.25	634.6±23.1	37.9±0.2	120.4±3.5
Sarin	16	7.446±0.004	41.18±0.71	87.97±2.84	608.6±13.9	37.5±0.1	116.1±4.0
Sarin+PB	16	7.442±0.008	41.85±0.76	86.82±1.09	601.8±11.8	37.5±0.1	108.7±4.9

Body mass was measured before animals were anesthetized for the rCBF or rCGU procedures. Mean blood pH and gases ($PaCO_2$; PaO_2), body temperature, and arterial blood pressure (MABP) were measured immediately before injection of the radioactive tracer. Statistical comparisons between treatment groups within a given time after treatment (weeks) indicated no significant differences.

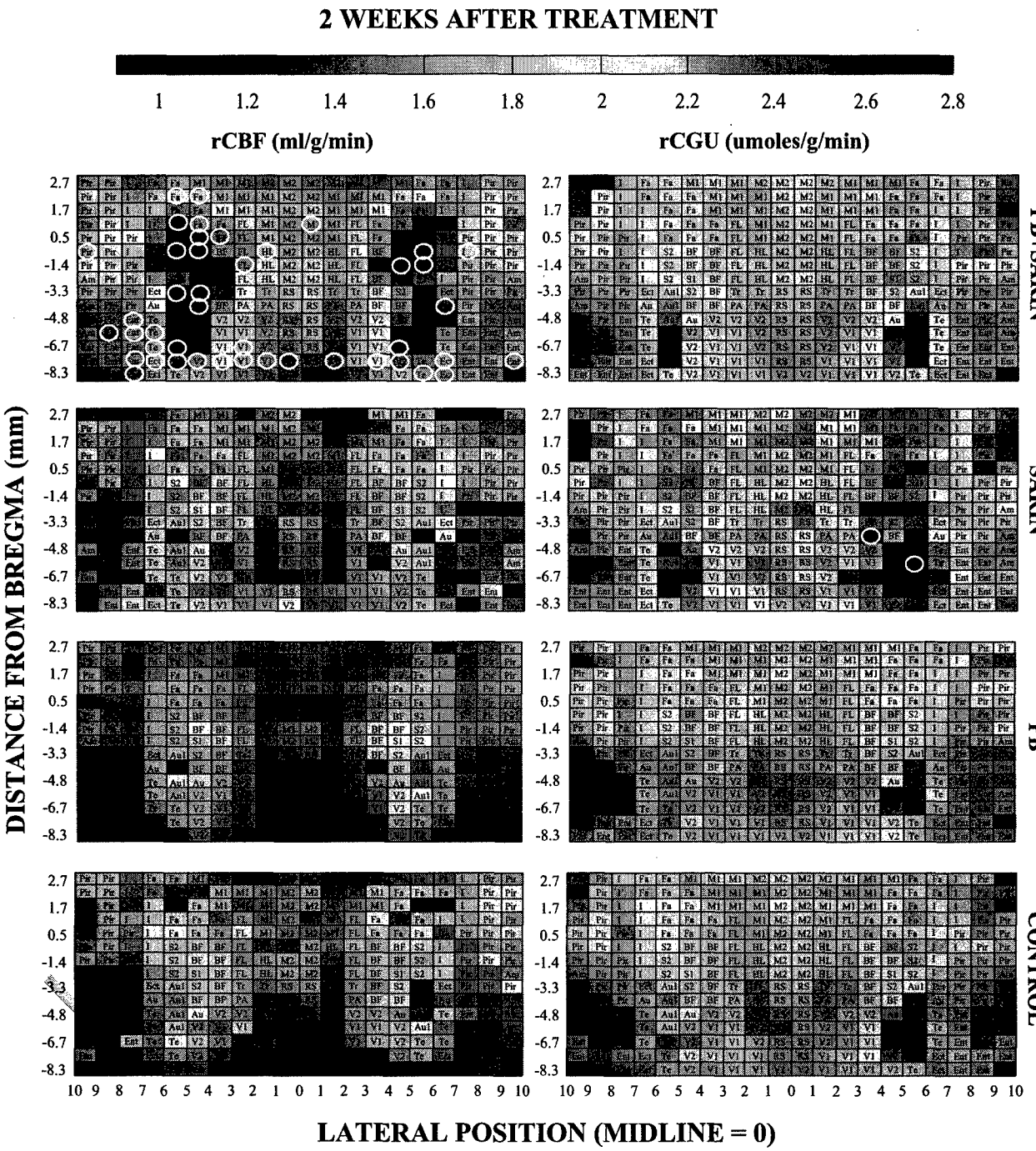

Fig. 2. Cerebral cortical rCBF (left panels) and rCGU (right panels) of animals studied 2 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=12; PB=10; Sarin=12; PB+Sarin=10. Number of animals in rCGU groups: Control=9; PB=6; Sarin=5; PB+Sarin=8. Regions are named according to Paxinos and Watson (Paxinos and Watson, 1998). Abbreviations: Am, amygdala; Au, auditory; Au1, primary auditory; BF, barrel field; Ect, ectorhinal; Ent, entorhinal; Fa, face area; FL, forelimb area; HL, hindlimb area; I, insular; M1, primary motor; M2, secondary motor; PA, parietal association area; Pir, piriform; RS, retrosplenial; S1, primary somatosensory; S2, secondary somatosensory; Te, temporal; Tr, trunk area; V1, primary visual; V2, secondary visual.

about 51% of baseline, which remained stable during the weeks of treatment and recovered after treatment ceased. Sarin produced a decrease in RBC AChE activity to about 33% of baseline, which remained stable during the treatment period and recovered following an irregular pattern with significantly lower values than controls during the third recovery week. The combination of PB and sarin also induced a significant depression of RBC AChE activity (27% of baseline), which persisted until the second week after treatment (Fig. 1).

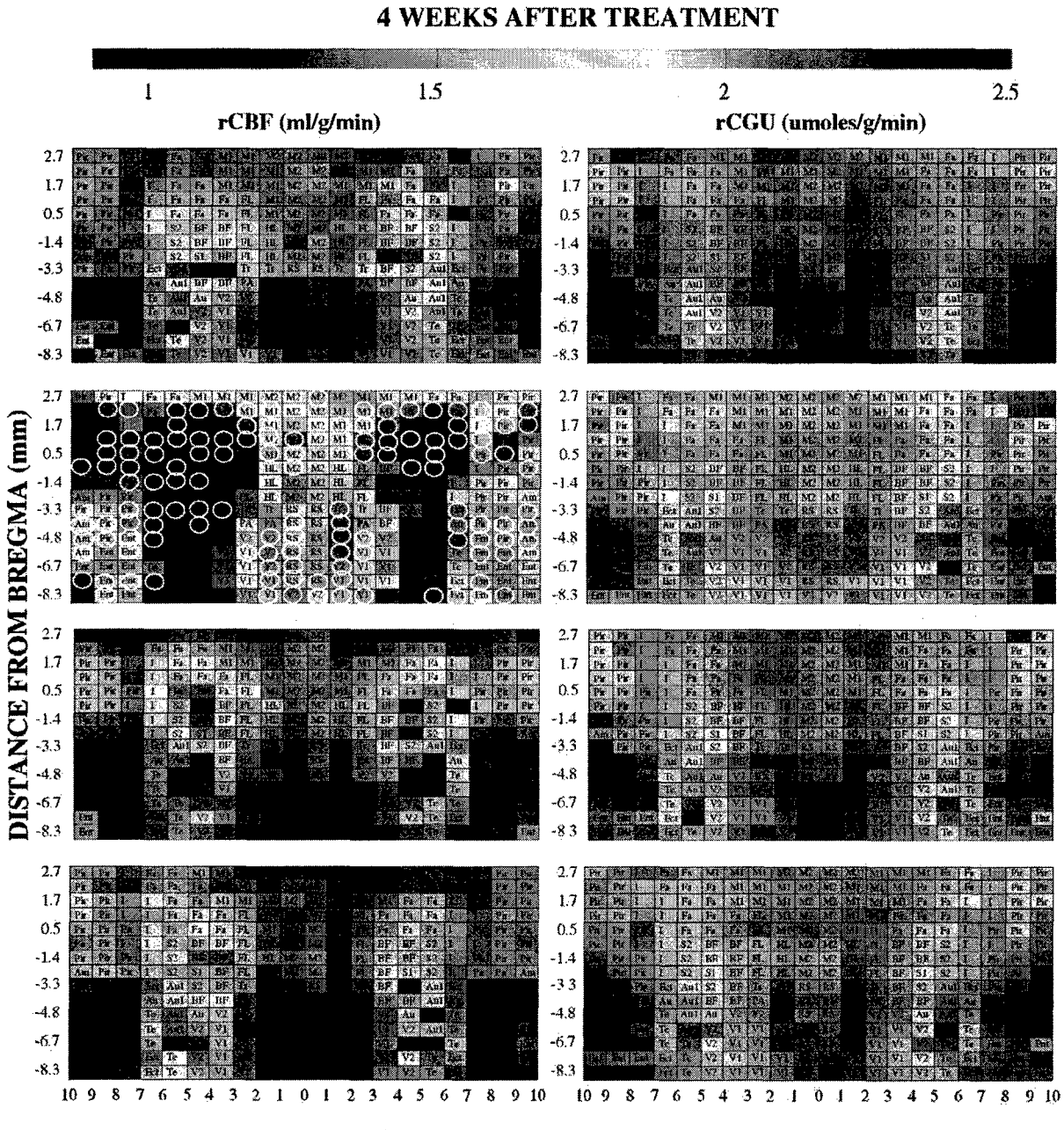

Fig. 3. Cerebral cortical rCBF and rCGU of animals studied 4 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=11; PB=8; Sarin=11; PB+Sarin=10. Number of animals in rCGU groups: Control=7; PB=7; Sarin=8; PB+Sarin=8. Regions are named according to Paxino and Watson (Paxinos and Watson, 1998). See abbreviations in Fig. 1 legend.

3.2. Arterial blood gases and pH, body temperature and mean arterial blood pressure

These variables, measured at the time of rCBF or rCGU measurements, did not show any significant differences with regard to controls for any of the experimental groups (Table 1). The expected increase in body mass with age was found between the three different times when rCBF and rCGU were measured, but no differences among groups were detected within a given time after treatment.

3.3. Cerebral blood flow and glucose utilization

Figs. 2–4 show, in three dimensional maps, the means of rCBF (left panels) and rCGU (right panels) of every location

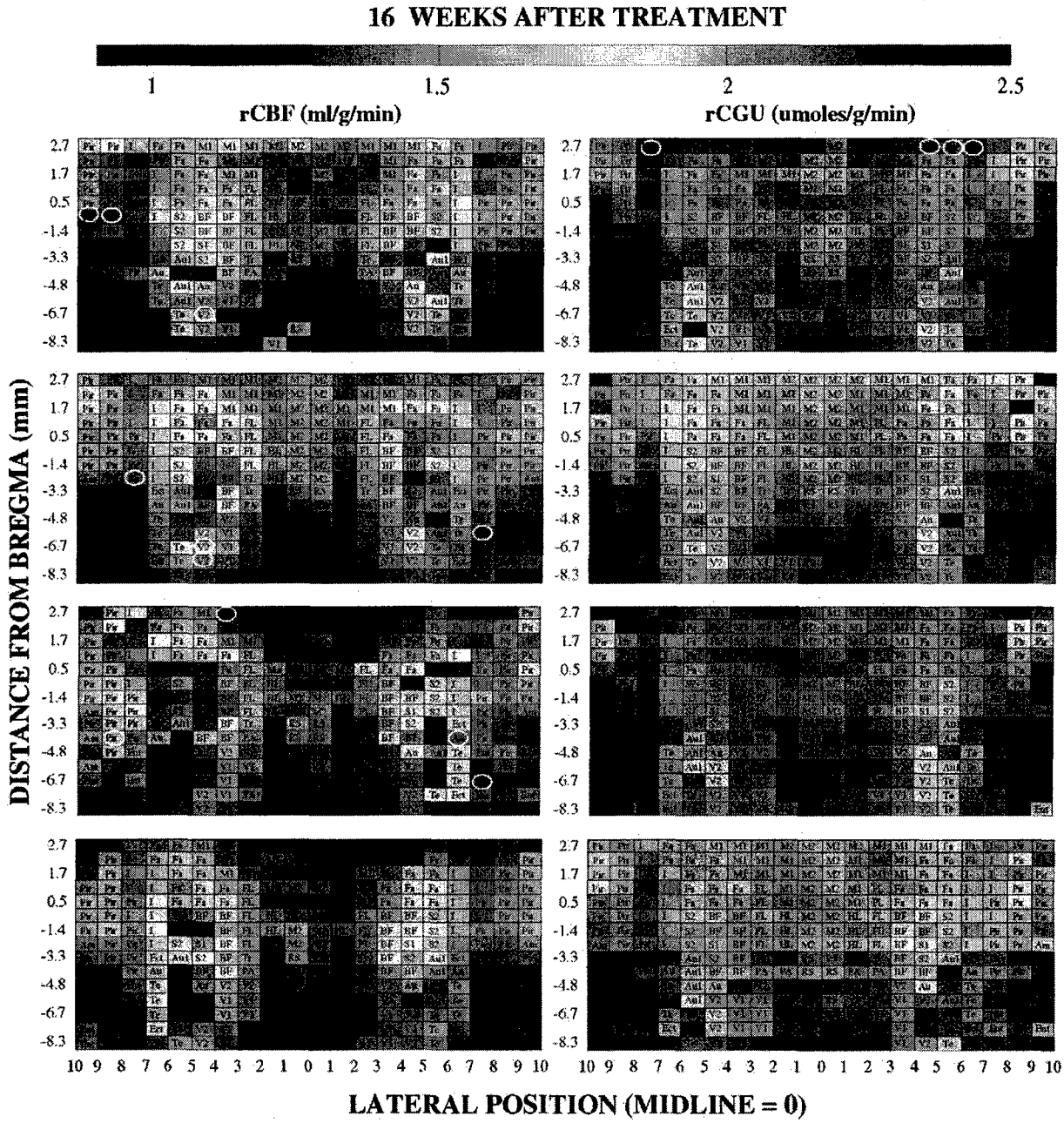

Fig. 4. Cerebral cortical rCBF of animals studied 16 weeks after discontinuation of treatment are displayed in three-dimensional maps in which the ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions relative to the midline. Means of rCBF or rCGU of the 300 cortical regions sampled in 15 coronal planes are coded on a common color scale (see color bar). Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts). Number of animals in rCBF groups: Control=11; PB=7; Sarin=8; PB+Sarin=11. Number of animals in rCGU groups: Control=5; PB=6; Sarin=7; PB+Sarin=5. Regions are named according to Paxinos and Watson (Paxinos and Watson, 1998). See abbreviations in Fig. 1 legend.

sampled. The ordinate represents position of regions (mm) along the rostro-caudal axis, with zero at bregma and values increasing towards the rostral end. The abscissa represents position of regions (mm) relative to the midline. Mean rCBF of every region is represented on a color scale. Statistical significance against the control group is indicated in these graphs by white ovals ($P<0.05$, Bonferroni adjusted for three contrasts).

Analysis of rCBF and rCGU in the control condition (no drug administration) indicated marked regional variations among locations within the cerebral cortex. In the case of rCBF cortical maps (Figs. 2, 3 and 4, bottom left panels) two rostral and one caudal clusters of locations with high rCBF were identified. The rostral paramedian high rCBF cluster included the face area (Fa), primary motor (M1), barrel field (BF) and secondary sensory (S2) areas, and the rostral lateral cluster was limited to the piriform region (Pir). The caudal high rCBF cluster included the primary auditory (Au1), temporal (Te), and secondary visual (V2) regions.

Cortical maps of rCGU resembled closely their rCBF counterparts in the caudal locations, but the rostral locations lacked a distinct high rCGU paramedian cluster resembling that described above for rCBF, while preserving a high rCGU cluster in the piriform region.

At 2 weeks after treatment (Fig. 2), significant changes in rCBF were only observed in animals treated with the combination of sarin+PB. The regions affected were located mostly on the neocortex (Fa, M2, S2, BF, FL, HL, Te, Au, Au1, V1, V2), with a few on Ent and Ect and only one on Pir. At 4 weeks after treatment (Fig. 3), the same general pattern was found in animals treated with sarin, with more significant locations in Pir, RS, and Am. Only few changes were found at 16 weeks post-treatment in the three experimental groups (Fig. 4).

In the case of rCGU cortical maps, very few and inconsistent statistically significant changes between experimental groups were found at each time after treatment (Figs. 2, 3 and 4, right panels).

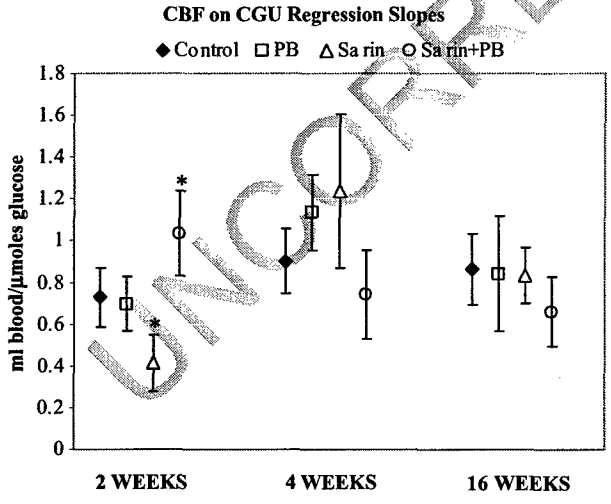

Fig. 5. The linear regressions of mean rCBF on mean rCGU for every region studied were calculated for every experimental group. The regression coefficients (slopes) and their 99% confidence intervals are shown. Statistical significance of differences between slopes of the three drug-treated groups against their respective controls for every time after treatment was assessed with the F ratio of the residual mean squares obtained when separate regressions were fitted for each condition to that obtained from a model in which a single pooled slope was fitted. The probability level at which differences were declared significant was set at 0.01 to compensate for the multiple comparisons performed. Asterisks indicate the groups in which the slopes differed significantly from controls.

3.4. Regression of rCBF on rCGU

Regression of rCBF on rCGU indicated slopes that were highly significantly different from zero with values ranging between 0.73–0.90 mL blood/µmol glucose in the control groups (Fig. 5). Comparisons of slopes of these regressions between drug treatment groups and controls indicated significant differences 2 weeks after treatment with an enhanced slope in animals treated with the combination of PB+sarin (1.04 mL blood/µmol glucose) and a decreased slope in the sarin group (0.41 mL blood/ µmol glucose). No statistically significant differences between slopes of drug treatment and control groups were found at 4 and 16 weeks after treatment.

4. Discussion

The experimental results yielded values of blood AChE inhibition consistent with this rat model, previously used to assess behavioral and neurological effects of sarin and PB (Scremin et al., 2003). Although brain AChE was not measured, previous data indicates that at the levels of blood AChE observed, significant brain AChE activity inhibition could be safely assumed (Shih, 1983; Shih et al., 1990; Roberson et al., 2001). The reason we measured AChE activity during treatment and shortly after is because the administration of the drugs did not continue after the initial 3 weeks. The aim of the paper was to look for delayed effects, i.e. beyond the period of AChE inhibition. A lack of any acute toxic effects during 3 weeks of sarin and PB administration, either alone or in combination, fulfilled the conditions required to model the potential low-level exposure of Persian Gulf War veterans. This model was, however, the "worse case" model for Persian Gulf War exposure scenario where veterans did not report any symptom of miosis, an initial sign of aerosol exposure.

Although it is generally assumed that rCBF and rCGU are valid correlates of brain function, it is important to measure both variables because there is ample evidence to indicate that rCBF, under the influence of vasoactive neurotransmitters, can be regulated independently from the levels of cerebral energy exchange (Scremin, 2003; Gulbenkian et al., 2001). Under both physiological and pathological conditions, many instances have been documented of a lack of correlation between rCBF and rCGU or

oxygen consumption (Gsell et al., 2000; Fox and Raichle, 1986). This is indeed the case in the current experiments. Large increases in rCBF were detected at 2 weeks in animals that received a combination of sarin and PB, and at 4 weeks in animals that received only sarin. In contrast, very few changes in rCGU were observed with these treatments and times after exposure.

The dissociation of rCBF and rCGU observed in the present experiments is similar to that observed immediately after administration of carbamate or OP cholinesterase inhibitors, known to enhance cerebral blood flow without a concomitant increase in rCGU or oxygen consumption, a phenomenon attributed to an excess of ACh at central sites with stimulation of muscarinic receptors (Scremin et al., 1982, 1988; Scremin, 1991; Blin et al., 1997). There are several possible causes for this phenomenon: 1) cholinergic stimulation primarily dilates cerebral blood vessels by a direct action on vascular smooth muscle without affecting neuronal function or metabolism, 2) cholinergic stimulation affects neuronal function with a very low (undetectable) metabolic cost, and the increase in rCBF is mediated by a neuronal non-metabolic mechanism, and 3) cholinergic stimulation affects neuronal function and enhances metabolism, but substrates other than glucose are used as fuels. The fact that neither glucose utilization nor oxygen consumption are enhanced by cholinergic agonists that induce large increases in rCBF argues against the last possibility (Scremin, 1991, 1993). It is well known that at appropriate doses, cholinergic agonists do affect the brain electrical activity and function (Lucas-Meunier et al., 2003). It is then possible that option 2 is more likely to be true. However, it is also possible that at low dose levels, a direct cerebrovascular effect of cholinergic agonists may be present without effects on nerve cells function. From the point of view of the objectives of this investigation, the important fact to consider is that the changes in nerve function, if any, were of a relatively transient nature since rCBF and rCGU changes were minimal at 16 weeks post-treatment. It is tempting to speculate that the early effects might have been due to residual inhibition of AChE in neurovascular compartments, that would dissipate at later times to explain the lack of effects at 16 weeks. However, this is merely speculative since we don't have any direct evidence of AChE inhibition in such compartments. It is by no means sure that the effects of these cholinesterase inhibitors observed after treatment, when AChE inhibition may be low or absent, are related to an immediate effect on the enzyme. We and other authors have observed delayed effects of low dose administration of AChE inhibitors beyond the period of enzyme inhibition. Moreover, similar blood AChE inhibition does not necessarily mean similar brain tissue inhibition. Sarin can cross the blood brain barrier but pyridostigmine does not, so the effects on blood AChE do not necessarily parallel changes in brain AChE.

The question remains as to why the effect of sarin when administered by itself was present at 4 weeks after treatment and not at 2 weeks. One possible explanation may be that in spite of residual AChE inhibition at 2 weeks, muscarinic receptor downregulation may have prevented the vascular effect from being expressed at this time. In support of this interpretation, we have previously detected significant downregulation of QNB binding 2 weeks after treatment with sarin but not with sarin+PB (Scremin et al., 2003). The difference in the effects of these two treatments may be related to the kinetics of central AChE inhibition, since occupation of peripheral AChE sites by PB may have displaced sarin towards central sites and enhanced ACh levels with regards to sarin alone, leading to the proposed muscarinic receptor downregulation at short times after treatment. These considerations are purely speculative, however, and elucidation of the mechanism of these late changes in rCBF with sarin alone or in combination with PB will require further experimentation.

Analysis of the regressions of rCBF on rCGU was carried out because the dependence of rCBF on rCGU levels is a well-known phenomenon that reflects the adjustments of blood flow, and hence of nutrients and oxygen supply, to the local levels of energy utilization. This is, however, not a constant, with variations known to occur following pharmacological interventions. Inhibition of AChE within the central nervous system is associated with enhancement of the slope of the rCBF/rCGU relationship (Scremin et al., 1993), while cholinergic muscarinic blockade with scopolamine has the opposite effect (Scremin and Jenden, 1996). The ratio of rCBF to rCGU may have significance in controlling the composition of the internal milieu of the brain and, thus, the excitability of nerve centers (Scremin, 2003). In the present experiments, animals that received sarin+PB manifested a significant enhancement in the rCBF/ rCGU slope 2 weeks after treatment, a phenomenon consistent with the hypothesis of residual AChE inhibition at this time. At the same interval after treatment, animals that had received sarin alone showed a significant decrease in the rCBF/rCGU slope, also in line with a downregulation of muscarinic receptors previously observed with sarin, but not sarin+PB at the same time after treatment in this experimental model (Scremin et al., 2003). The differential effect of the two treatments could thus be explained by the predominance of receptor downregulation that may have prevented the effect of excess ACh due to residual AChE inhibition, as discussed above for the differential effect on rCBF of both treatments.

In conclusion, the changes in rCBF and rCGU observed in the present experiments are consistent with a combination of residual AChE inhibition and downregulation of muscarinic receptors. The changes were not present at 16 weeks after treatment, a fact that does not support the hypothesis that low-level sarin or PB could elicit permanent changes in the central nervous system.

5. Uncited reference

Sidell et al., 1974

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